Endocrine Journal 2009, **56** (4), 619-624

NOTE

A Novel Heterozygous Mutation of Steroidogenic Factor-1 (SF-1/Ad4BP) Gene (*NR5A1*) in a 46, XY Disorders of Sex Development (DSD) Patient without Adrenal Failure

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Abstract. Steroidogenic factor-1 [(SF-1/Ad4BP) (MIM184757)] is a nuclear receptor that regulates multiple genes involved in adrenal and gonadal development, steroidogenesis, reproduction, and other metabolic functions. Initially, mutations of SF-1/Ad4BP gene (*NR5A1*) in humans were identified in two 46, XY female patients with adrenal insufficiency and gonadal dysgenesis. However, recent studies have revealed that heterozygous mutations are more frequently found in 46, XY disorders of sex development (DSD) patients without adrenal failure than in 46, XY DSD patients with adrenal failure. We encountered a Japanese female patient of 46, XY DSD without adrenal failure and identified a novel mutation (V41G) of *NR5A1*. Functional analysis revealed that this mutant protein could not activate *CYP19* promoter, indicating loss of function. In conclusion, we add a novel mutation of *NR5A1* in 46, XY DSD patient without adrenal failure.

Key words: SF-1/Ad4BP, *NR5A1*, 46 XY DSD, Adrenal failure

(*Endocrine Journal* **56**: 619-624, 2009)

STEROIDOGENIC factor-1 [(SF-1/Ad4BP) (MIM184757)] is a nuclear receptor that was first identified following the search for a common regulator of the cytochrome P450 steroid hydroxylase family of enzymes [1-3]. SF-1/Ad4BP regulates multiple genes involved in adrenal and gonadal development, steroidogenesis, reproduction, and other metabolic functions [1-3]. In mice, SF-1/Ad4BP is expressed early in the urogenital ridge and continues to be expressed in the developing adrenal, gonad, ventromedial hypothalamus and pituitary [4]. Homozygous knockout mice result in gonadal and adrenal agenesis, present of Mullerian structure and abnormalities of the hypothalamus and pituitary gonadotrope. Heterozygous mice have a milder phenotype including an impaired adrenal stress response and reduced testicular size [5].

In human, initially, mutations of SF-1/Ad4BP gene (*NR5A1*) were identified in two 46, XY female patients with adrenal insufficiency and gonadal dysgenesis [6, 7]. The first mutation (G35E) was a heterozygous mutation, which occurred in the P-box of the first zinc finger domain required for the DNA-binding [6, 8]. The second mutation was a homozygous missense mutation (R92Q) that disrupts the A-box secondary DNA binding domain [7]. However, recent several studies have demonstrated that heterozygous mutations are more frequently identified in 46, XY disorders of sex development (DSD) patients without adrenal failure rather than in 46, XY DSD patients with adrenal failure [3, 9-17]. Therefore, the testis is likely to be more sensitive to partial loss of SF-1/Ad4BP function than the adrenal gland in human. In regard to females with heterozygous *NR5A1* mutations, so far seven individuals have been reported [13-18]. Among them, only one patient developed adrenal failure, but she had apparently normal ovarian function [18]. The remaining individuals were mothers of 46, XY DSD patients and they did not show any symptoms of adrenal and ovarian failure [13-17]. Indeed, ovarian development during development and at birth is relatively

Received Dec. 22, 2008; Accepted Feb. 27, 2009 as K08E-380
Released online in J-STAGE as advance publication Mar. 24, 2009
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preserved in ovary-specific Sf-1 knockout mice compared to testes in testes-specific Sf-1 knockout mice [19]. These findings indicate that only one intact SF-1/Ad4BP is sufficient for normal ovarian development.

Here, we experienced a Japanese 46, XY DSD patient and found a novel mutation (V41G) of *NR5A1*. *In vitro* study demonstrated this mutation lost its function.

Methods

DNA amplification and sequence analysis

Informed consent to participate in the study was obtained from the patient and parents. The ethical committee of Hokkaido University School of Medicine approved this study. Genomic DNA was extracted from peripheral leukocytes and each exon of *NR5A1* was amplified by polymerase-chain-reaction (PCR) according to a previous report [10]. After amplification, the PCR products were purified and sequenced directly using an ABI PRISM Dye Terminator Cycle Sequencing Kit and an ABI 373A automated fluorescent sequencer.

Mutant SF-1 cDNA construction and plasmid construction

Human SF-1/Ad4BP cDNA was inserted into pcDNA 3.1 (WT-SF-1/Ad4BP). The mutant cDNA was created by site-directed mutagenesis using an overlapping PCR strategy and was designated MT-SF-1/Ad4BP. The mutation was verified by direct DNA sequencing. The human cytochrome P450arom gene (*CYP19*) promoter luciferase plasmid was used for analysis of SF-1/Ad4BP function as described previously [20, 21]. This construct was designated pGL3-CYP19.

Cell culture

COS cells were obtained from American Type Cell Culture and grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum.

Transient gene expression

In order to assay *CYP19* promoter activity, COS cells were plated in 6-well plates, grown to 70% confluency and transiently transfected by lipofectamine with either (1) empty expression vector (pCDNA3, 0.2 μg); (2) WT-SF-1/Ad4BP (0.2 μg); (3) MT- SF-1/Ad4BP (0.2 μg); (4) WT-SF-1/Ad4BP (0.2 μg) plus MT-SF-1/Ad4BP (0.2 μg); (5) WT-SF-1/Ad4BP (0.2 μg) plus MT-SF-1/Ad4BP (0.4 μg); or (6) WT-SF-1/Ad4BP (0.2 μg) plus MT-SF-1/Ad4BP (1.0 μg) together with pGL3-CYP19 (0.4 μg). Cell extracts were prepared 48 hours after transfection and luciferase assays were performed. Luciferase measurements were divided by the respective β-galactosidase activity to control for transfection efficiency. The mean of each triplicate reaction was expressed as a percentage of the empty vector control to allow comparison of data from different experiments. Data are presented as means±S.D.

Table 1. Endocrinological findings

Blood hormone value	Basal	Peak
LH (mIU/mL)[a]	14.35	59.81
FSH (mIU/mL)[a]	41.1	59.85
Estradiol (pg/ml)[b]	<10	<10
Testosterone (ng/ml)[b]	0.78	2.57
ACTH (pg/ml)	58.5	
Cortisol (mg/dl)[c]	14.8	20.12
17-OHP (ng/dl)[c]	2.3	5.4

a, GnRH test(100mg). b, Three-day hCG stimulation (5000IU). c, Cortisol and 17-hydroxyprogesterone after acute stimulation with 250 mg ACTH intravenous injection.

A report of case

A Japanese patient was born after 40 weeks of gestation by normal vaginal delivery and was the first child of nonconsanguineous parents. She had no siblings, and her parents were healthy. Her birth weight was 3490 g and length was 49.5 cm. At birth, clitoromegaly was noticed; however, further medical examination was not performed. Thus, the patient was reared as a female. At 12 years of age, she was referred to our hospital because of no development of secondary sexual characteristics and clitoromegaly. Her height was143 cm and body weight was 35 kg. Breast development was at Tanner stage I, and pubic hair development at Tanner stage I. She had clitoromegaly (~2.2 cm), but no posterior labial fusion and the vaginal and the urethral orifices were separated. Presumed gonads were palpable bilaterally in the in-

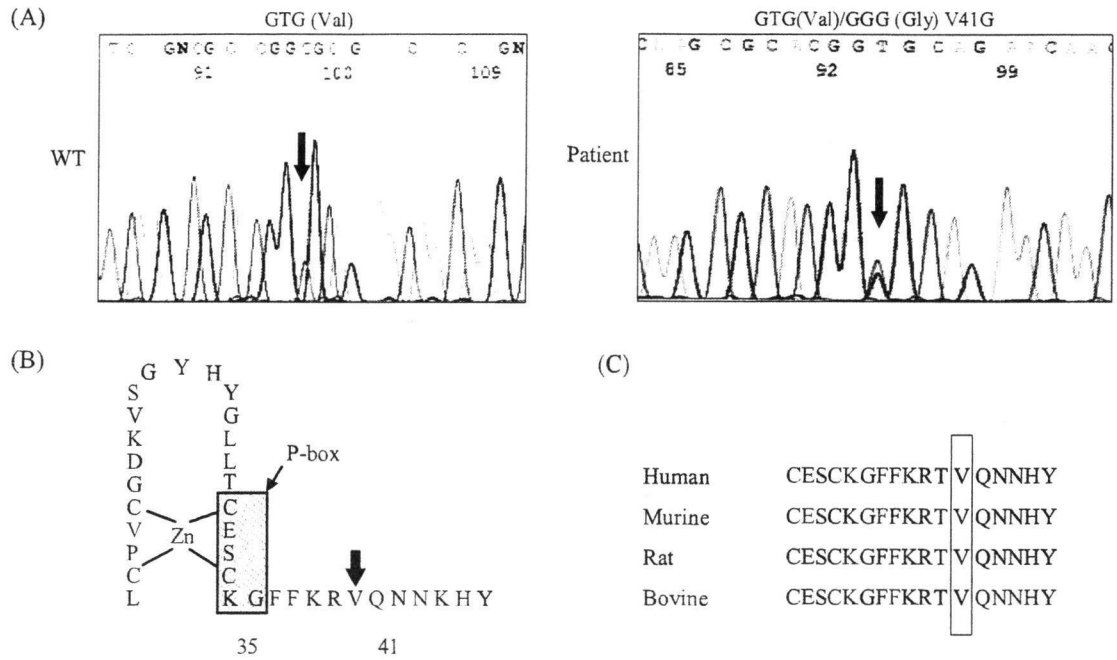

Fig. 1. (A) Sequence analysis demonstrated a T to G transition. This change substitutes glycine for valine at residue 41 in the first zinc finger domain of SF-1/Ad4BP as denoted by an arrow. (B) A part of the amino acid sequence of the first zinc-finger domain of SF-1/Ad4BP protein is shown. A hatched box indicates P-box. (C) Part of the amino acid sequence of the first zinc finger domain of SF-1/Ad4BP is shown. A box indicates the conserved valine residue at codon 41.

guinal region. Skin pigmentation was not observed. She had no episode of adrenal insufficiency during her life. Her karyotype was 46, XY. Her endocrinological evaluation is summarized in Table 1. Her serum estradiol concentration was less than 10 pg/ml. Basal serum testosterone concentration was 0.78 ng/ml and after human chorionic-gonadotropin (hCG) stimulation (5000 IU intramuscularly daily for 3 days), serum testosterone increased up to 2.57 ng/ml. Basal gonadotropin levels were elevated [follicle stimulating hormone (FSH) 41.1 mIU/ml, luteinizing hormone (LH) LH 14.35 mIU/ml].

Her basal cortisol and adrenocorticotropin (ACTH) levels were within normal range (14.8 μg/dl and 58.5 pg/ml, respectively). After ACTH stimulation, serum cortisol increased up to 20.1 μg/dl without any abnormal accumulation of adrenal steroid precursors. Pelvic magnetic resonance imaging demonstrated no uterus or vaginal pouch. Laparoscopy did not show any Mullerian derivatives. Genitoplasty and gonadectomy were performed. Histological examination revelaed dysgenetic testis. Microscopic examination showed that seminiferous contained Sertoli cells, but rare germ cells, and loose interstitium had a few clusters of Leydig cells.

She is now 19 years-old and being treated with estrogen supplementation. Her height is 161 cm and body weight is 42 kg. Her basal cortisol and ACTH at 9:00 A.M. are 7.5 μg/dl and 27.5 pg/ml. Until this time, she has not developed adrenal failure.

Results

Sequencing analysis of *NR5A1* revealed a heterozygous point mutation in exon 3 at codon 41 [GTG (Val) to GGG (Gly)] (Fig. 1A). Fifty normal Japanese subjects did not show this base change. This mutation is present in the first zinc finger domain (Fig. 1B) and this valine at residue 41 is well conserved in different species (Fig. 1C). Her parents were not subjected to DNA analysis.

In vitro transfection study demonstrated that WT-SF-1/Ad4BP activated the *CYP19* promoter activity, whereas MT-SF-1/Ad4BP did not (Fig. 2). Cotransfection of mutant with WT-SF-1/Ad4BP did

not show a dominant negative effect even when 5:1 ratios of MT:WT-SF-1/Ad4BP were transfected (Fig. 2).

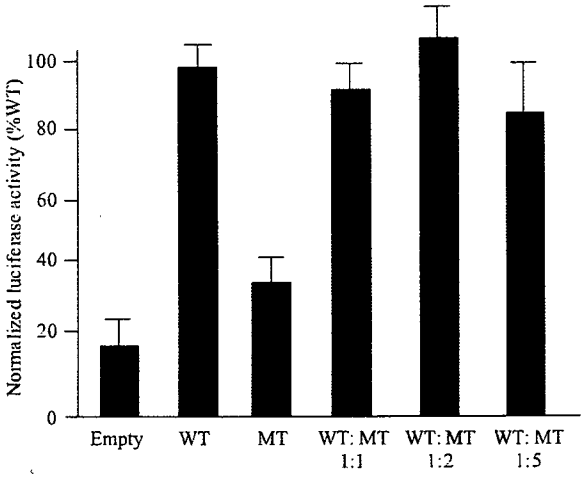

Fig. 2. Transactivation function of WT-SF-1/Ad4BP and MT-SF-1/Ad4BP. While cotransfection of WT-SF-1/Ad4BP with *CYP19* promoter stimulated the luciferase reporter gene relative to the empty vector, MT-SF-1/Ad4BP did not. Transfection of increasing amounts of MT-SF-1/Ad4BP did not impair the transactivation capacity of the wild-type protein, suggesting no dominant negative effect of the mutant protein. Data are presented as means±S.D.

Discussion

We identified a novel mutation of V41G in a 46, XY DSD patient without adrenal insufficiency. *In vitro* promoter assay demonstrated that V41G protein lost activating function. So far C33S and G35E mutations were identified in the P-box region of the first zinc finger domain, and these two mutants lost a DNA-binding activity [6, 17]. As shown in Figure 1, the V41 residue is located near the P-box region in the first zinc finger domain, and is highly conserved among different species. Therefore, although we did not analyze DNA binding, our mutant protein would affect DNA binding, resulting in loss of function.

Despite ambiguous genitalia, our patient showed low but increased serum testosterone level after hCG stimulation at 12 years of age. These findings might be explained by insufficient testosterone production, especially during the development of the external genitalia. Most patients with SF-1/Ad4BP mutation demonstrated severe defect in testosterone production. However, low but detectable testosterone levels basal or after hCG stimulation were observed in five patients [9, 12, 14, 15, 17]. Among these patients, one had very mild phenotype of penoscrotal hypospadias and was raised as a male [15]. Consistent with the mild phenotype, the mutation (L437Q) of this patient retained partial function *in vitro*. The authors suggest the genotype may partly explain the mild phenotype. However, the other patient with increased testosterone (2.5 ng/ml) level after hCG stimulation demonstrated significant undervirilization [17]. This patient had C33S mutation with complete loss of function. Furthermore, Coutant *et al.* [14] have reported two siblings caused by a severe SF-1/Ad4BP mutation (c.536delC), who lack the ligand-binding domain and the activation function 2 domain. The elder 46, XY female showed ambiguous genitalia and elevated testosterone (2.2 ng/ml) level in neonatal period, which led to a presumable diagnosis of partial androgen insensitivity syndrome. By contrast, the second child of 46, XY female had less virilized genitalia than the older child and her testosterone production was severely defect. This report indicated that the difference of the phenotypes and Leydig cell function existed even in the familial case. Accordingly, not only the genotype of SF-1/Ad4BP, but also other genetic or environmental factors seem to affect testosterone production of Leydig cell during the critical period of the development of the male external genitalia. This must be further studied.

So far, 16 of the 46, XY DSD patients without adrenal failure caused by SF-1/Ad4BP mutations were reported (9-17). Lin *et al.* [15] have reported four 46, XY DSD patients with SF-1/Ad4BP mutations among 30 patients with 46, XY DSD. Köhler *et al.* [17] have reported 5 patients with SF-1/Ad4BP mutations in a cohort of 27 patients with 46, XY DSD. Four Japanese 46, XY DSD patients without adrenal failure caused by SF-1/Ad4BP mutations have already been reported [10, 12, 13]. Thus, it is plausible that SF-1/Ad4BP mutations are more frequent than previously suspected causes of 46, XY DSD in Japan. To clarify this hypothesis, a systemic cohort of 46, XY DSD patients throughout Japan is necessary.

In conclusion, we identified a novel mutation of *NR5A1* in a Japanese 46, XY DSD patient without adrenal failure.

Acknowledgments

We thank Dr. Ken-Ichirou Morohashi, Department of Molecular Biology Faculty of Medical Sciences, Kyushu University, for providing us with the human Ad4/BP expression vector, Dr. Toshihiko Yanase, Department of Medicine and Bioregulatory Science, Graduate School of Medical Sciences, Kyushu University, for providing human *CYP19* promoter vector and Dr. Tomonobu Hasegawa, Department of Pediatrics, Keio University School of Medicine, for PCR primers for *NR5A1*.

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Long-Term Outcome of Vaginoplasty With the Bilateral Labioscrotal Flap

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Purpose: We report the long-term outcome of vaginoplasty with the bilateral labioscrotal flap with special emphasis on vaginal stenosis.

Materials and Methods: Of 23 children with ambiguous genitalia and low vaginal entry who underwent vaginoplasty between January 1985 and July 2003 with the bilateral labioscrotal flap 13 followed more than 5 years after surgery who were 10 years old or older at the most recent evaluation were included in this long-term outcome study. Vaginal caliber was estimated according to a previously described assessment system adopted for vaginoplasty results.

Results: The underlying disease was congenital adrenal hyperplasia in 11 cases, mixed gonadal dysgenesis in 1 and ovotesticular sexual development disorder in 1. Mean age at vaginoplasty and at the most recent evaluation was 3.8 (range 2.0 to 12.9) and 14.6 years (range 10.9 to 21.5), respectively. Vaginal caliber at the most recent evaluation was adequate in 6 patients (46%), stenotic in 5 (39%) and strictured in 2 (15%). Three of the 7 patients diagnosed with stricture or stenosis were diagnosed at age less than 12 years. One of these patients diagnosed with stricture was treated with dilation and the other 2 patients were observed. These patients had no trouble with menstruation. Four patients diagnosed with stricture or stenosis at age 14 years or older were treated surgically with dilation in 1 and perineal flap vaginoplasty in 3. They showed adequate vaginal caliber at 3 to 31 months of followup. In 7 patients evaluated at the beginning of puberty and several years later vaginal caliber had enlarged in 5 but remained unchanged in 2.

Conclusions: To our knowledge this is the first report of the long-term outcome of vaginoplasty with the bilateral labioscrotal flap. Although vaginal stenosis/stricture was observed at puberty in about half of the patients, severe stricture was uncommon. Serial evaluation for vaginal stenosis/stricture at the beginning of puberty for menstruation and several years later for vaginal intercourse is recommended in patients treated with vaginal reconstruction.

Key Words: sex differentiation disorders; surgical flaps; vagina; constriction, pathologic; Japan

In patients with ambiguous genitalia assigned a female gender feminizing genitoplasty is usually performed early in life. This procedure is vital to facilitate a favorable genital appearance, which is a fundamental factor in childhood gender and psychosexual development, normal menstruation and satisfactory intercourse in adult life with nonpainful penetration and orgasmic sensation.

Various surgical techniques for female reconstruction of ambiguous genitalia have been reported to date. Ana-

0022-5347/09/1824-1876/0
THE JOURNAL OF UROLOGY®

Vol. 182, 1876-1881, October 2009
Printed in U.S.A.
DOI:10.1016/j.juro.2009.02.090

lyzing the long-term outcome is a crucial point in evaluating these surgical techniques. However, reports of long-term outcomes after feminizing genitoplasty are scarce. Most of the previous literature focusing on long-term followup studies documents a poor outcome with a high incidence of stenosis at the vaginal introitus and the need for further intervention.[1–6]

While flap vaginoplasty with the perineal skin flap has been commonly used to exteriorize the low entry vagina,[7] we have performed vaginoplasty with the bilateral labioscrotal flap to avoid stenosis because the labioscrotal skin is more elastic and more easily elongated than the perineal skin.[8] We report the long-term outcome of vaginoplasty with the bilateral labioscrotal flap with special emphasis on vaginal stenosis.

MATERIALS AND METHODS

From January 1985 to July 2003, 23 children with ambiguous genitalia and low vaginal entry, defined by Hendren and Donahoe as the vagina and urethra coming together in the urogenital sinus distal to the external urethral sphincter,[9] underwent vaginoplasty with the bilateral labioscrotal flap. Genetics, hormonal response and anatomical status in all patients with ambiguous genitalia were evaluated and a diagnosis was made before genital reconstruction.

Figure 1 shows vaginoplasty with the bilateral labioscrotal flap.[10] After reduction clitoroplasty with the corporeal body resected the bilateral labioscrotal flap was designed by incising along the opening of the urogenital sinus and the incision was extended to the bilateral lower third of the labioscrotal folds to make an M-shaped skin incision (fig. 1, *A* to *F*). A vertical incision at the 6 o'clock position of the urogenital sinus was made to reach the vaginal introitus. The 2 medial edges of the labioscrotal flap were approximated and inlayed as 1 body into the posterior edge of the vaginal introitus (fig. 1, *G*). After completing vaginal exteriorization minor labia were created using the bisected dorsal clitoral foreskin (fig. 1, *H*). A urethral catheter and vaginal packing were placed during the operation and removed a few days later.

To assess the long-term outcome we retrospectively reviewed the medical charts of patients followed more than 5 years after surgery who were 10 years old or older at the most recent evaluation. Of the 23 patients 13 who met these criteria were included in this study.

Vaginal caliber was evaluated using a Hegar dilator under anesthesia in 2 patients and while awake in 11 (fig. 2). The Hegar dilator was gently inserted with minimal force when the vaginal caliber was assessed using anesthesia. Vaginal caliber was defined when any resistance was felt at insertion. Vaginal caliber was classified according to the assessment system adopted for vaginoplasty results reported by Gupta et al with modification.[10]

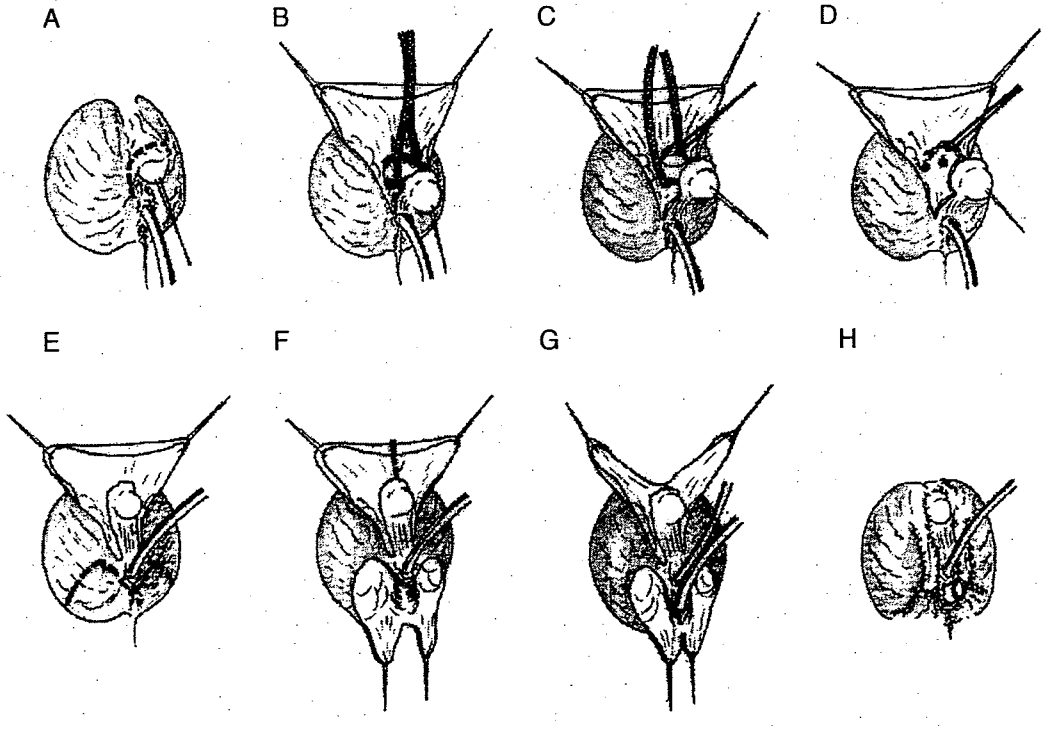

Figure 1. For reduction clitoroplasty semicircumcoronal incision (dashed line) is designed (*A*) and clitoral body is degloved (*B*). Neurovascular bundles are isolated (*C*) and clitoral body is resected, preserving neurovascular bundles and glans clitoris (*D*). After glans clitoris is anchored to mons pubis bilateral labioscrotal flap (dashed line) is designed (*E*). Labioscrotal flap is dissected from urogenital sinus posterior (*F*), approximated in midline (*G*) and inlayed into incised urogenital sinus in same manner as perineal inverted U-shaped flap (*H*). For labioplasty dorsal foreskin is bisected (*G*) and draped around glans clitoris toward new vaginal introitus (*H*).

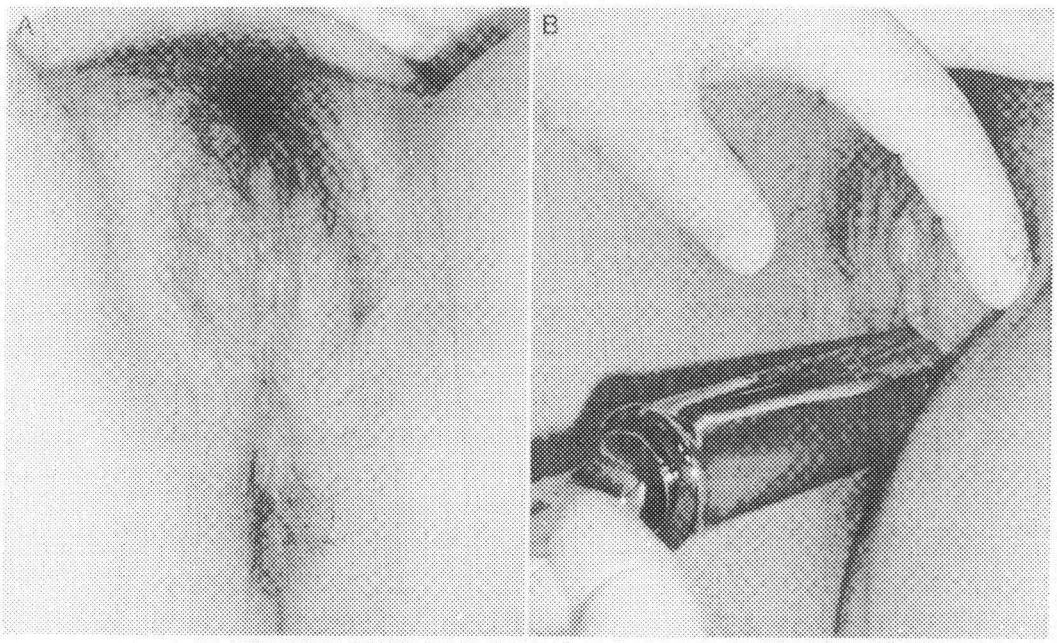

Figure 2. Cosmesis at puberty of genitalia reconstructed by vaginoplasty with labioscrotal flap (*A*) with vaginal caliber estimated using Hegar dilator (*B*).

The vagina was considered strictured when vaginal diameter was less than Hegar size 10, stenotic when diameter was 10 to 16 and adequate when it was greater than 16.

Changes in vaginal caliber were examined between the beginning of puberty at ages 10 to 13 years and several years later at age 14 years or later. When vaginal caliber classified by the assessment system had improved and/or there was an enlargement of a Hegar size of 5 or more between the 2 evaluations, it was defined as enlarged.

RESULTS

The underlying disease was congenital adrenal hyperplasia in 11 cases, mixed gonadal dysgenesis in 1 and ovotesticular sex development disorder in 1. Age at vaginoplasty was 2.0 to 12.9 years (mean 3.8). Age at the most recent assessment before the secondary intervention if necessary was 10.9 to 21.5 years (mean 14.6). Mean followup was 10.8 years (range 6.3 to 18.9) after vaginoplasty.

No hair bearing was observed in the labioscrotal flap used for vaginoplasty at puberty in all patients. Vaginal caliber at evaluation was diagnosed as adequate in 6 patients (46%), stenotic in 5 (39%) and strictured in 2 (15%). Stenosis/stricture occurred at the flap/vagina junction in patients with the diagnosis of stenosis or stricture.

Of 7 patients diagnosed with stenosis or stricture the diagnosis was made at the time of regular evaluation at puberty in 6 and by hematocolpos in 1. Three of these 7 patients were younger than 12 years, including 1 diagnosed with stricture who was treated with dilation. The other 2 patients, who were diagnosed with stenosis, were observed without any surgical procedure until reevaluation several years later because vaginal caliber was sufficient for menstruation. Four patients diagnosed with stenosis or stricture at age 14 years or older were treated surgically with dilation in 1 and perineal flap vaginoplasty in 3 (see table).

The 3 patients diagnosed with stricture or stenosis at age less than 12 years, including 1 treated with dilation at younger than 12 years and followed for 51 months after dilation, had no trouble with menstruation. Four patients treated at age 14 years or older had an adequate vaginal caliber at 3 to 31 months of followup (mean 20).

Serial evaluation of vaginal caliber was performed at the beginning of puberty and several years later in 7 patients. Vaginal caliber enlarged in 5 patients from stenotic to adequate in 4 and from Hegar size 20 to 25 in 1, which was within the definition of adequate. It remained unchanged in 2 patients (stenotic and strictured in 1 each) during 24 to 132 months (mean 51.3) of observation (fig. 3).

DISCUSSION

Various surgical procedures for feminizing genitoplasty have been reported to date. In patients with ambiguous genitalia and a low vaginal entry flap vaginoplasty with a perineal inverted U-shaped flap[6] was a standard feminizing genitoplasty procedure for many years. Despite an excellent cosmetic outcome in the short term few long-term outcomes of

Patient characteristics and long-term outcome of vaginoplasty with bilateral labioscrotal flap

Underlying Disease	Age (yrs)		Vaginal Caliber (Hegar size)	Treatment	Posttreatment Followup (mos)	Posttreatment Vaginal Caliber (Hegar size)
	At Surgery	At Evaluation				
Congenital adrenal hyperplasia	2.2	11.5	Adequate (18)			
Congenital adrenal hyperplasia	2.1	13.8	Adequate (30)			
Congenital adrenal hyperplasia	3.5	13.9	Adequate (24)			
Congenital adrenal hyperplasia	4.8	14.6	Adequate (20)			
Congenital adrenal hyperplasia	3.1	14.7	Adequate (20)			
Mixed gonadal dysgenesis	2.6	21.5	Adequate (18)			
Congenital adrenal hyperplasia	2	10.9	Stenotic (13)	Observation		No trouble at menstruation
Ovotesticular sexual development disorder	3.2	11.6	Strictured (less than 10)	Dilation	51	No trouble at menstruation
Congenital adrenal hyperplasia	5.2	11.8	Stenotic (16)	Observation		No trouble at menstruation
Congenital adrenal hyperplasia	2	14.0	Stenotic (11)	Flap vaginoplasty	25	Adequate (22)
Congenital adrenal hyperplasia	3.2	15.6	Strictured (less than 10)	Dilation	20	Adequate (25)
Congenital adrenal hyperplasia	2.5	16.3	Stenotic (12)	Flap vaginoplasty	31	Adequate (22)
Congenital adrenal hyperplasia	12.9	19.2	Stenotic (15)	Flap vaginoplasty	3	Adequate (18)

this procedure were reported. The literature describing long-term outcome shows that vaginal stenosis was noted in 36% to 94% of these patients at puberty or later.[1–6] The cause of these poor outcomes might be related to the quality of the posteromedian perineal skin, which usually lacks elasticity and sufficient vascularity.

The M-shaped labioscrotal flap was originally reported by Hecker to improve the outcome of vaginoplasty.[11] We used that method with modifications because a labioscrotal flap is more elastic than a perineal skin flap. By approximating the well vascularized flap, which is supplied by the superficial perineal branches of the pudendal artery, in accordance with the distance to the 6 o'clock position of the vaginal introitus the elastic subcutaneous tissue of these flaps could be inlayed without undue tension. Recently another procedure using labioscrotal island flaps was reported to enlarge the vaginal introitus and to decrease tension on the mucocutaneous anastomosis.[12] Although the short-term outcome of vaginoplasty with the bilateral labioscrotal flap was reported,[7] to our knowledge the long-term outcome has not been reported to date.

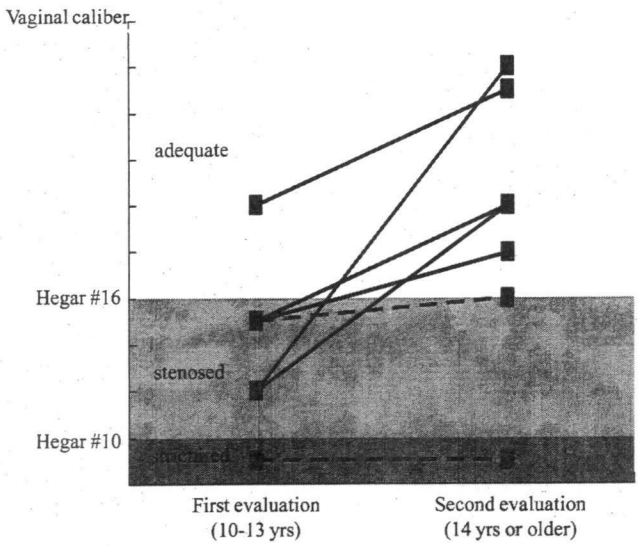

Figure 3. Changes in vaginal caliber between first evaluation at beginning of puberty and second evaluation in 7 patients (rectangles) during 24 to 132 months (mean 51.3) of observation.

In this study 7 of 13 patients (54%) showed vaginal stenosis/stricture at long-term followup, of whom 5 were treated surgically with dilation (2) and flap vaginoplasty (3). Although vaginal stenosis/stricture was observed at puberty in about half of the patients, severe stricture was uncommon.

A reason for this relatively high stenosis/stricture rate might be related to the timing of evaluation. In the original study of the assessment system reported by Gupta et al 19 girls older than 14 years were evaluated.[10] In our study patients 10 years old or older were enrolled and 3 of 7 with a diagnosis of stenosis or stricture were younger than 12 years. Accordingly there might be a risk of over diagnosis of vaginal narrowing using the assessment system in such young patients, who would be expected to have a wider vaginal caliber with time under estrogen exposure during the next several years (fig. 3). Therefore, 2 patients diagnosed with stenosis at age less than 12 years were observed without any surgical procedure until the second evaluation.

Evaluating the reconstructed vagina is not necessary until the onset of puberty since a functional vagina becomes important at puberty or later. However, evaluation at puberty is mandatory in patients who underwent feminizing genitoplasty during childhood because of its high incidence of long-term complications.[1–6] We usually perform 2 evaluations

of vaginal caliber, including 1 at the beginning of puberty for menstruation and the other several years after the first evaluation to assess the ability to achieve penetrating nonpainful vaginal intercourse. The outcome of the most recent evaluation before the secondary intervention in each patient was used in this study. Accordingly age at evaluation varied depending on the purpose.

At the first evaluation surgery is not indicated unless severe vaginal stricture that might cause hematocolpos is identified. If severe stricture is identified at the beginning of puberty, dilation is performed using anesthesia. When stenosis/stricture is observed late in puberty, secondary intervention may be indicated under hormonal stimuli. While vaginal dilation was performed emergently in 1 patient in whom vaginal stricture was detected by hematocolpos, we perform flap vaginoplasty as elective secondary intervention in patients diagnosed with stenosis or stricture at the second evaluation. In patients treated with secondary intervention vaginal caliber is reassessed 3 to 6 months after secondary intervention with the patient awake.

Under hormonal stimuli periodic dilation performed by the patient, if necessary, is also more likely to be successful at puberty with motivation.[3,5] Periodic self-dilation by patients at puberty or later is indicated when vaginal caliber is adequate with or without secondary intervention but it does not seem to be sufficient for vaginal intercourse.

Another reason for vaginal stenosis/stricture might be that our procedure involved more suture lines than conventional flap vaginoplasty because the bilateral labioscrotal flap was approximated in the midline. According to the long-term outcome of various vaginoplasty techniques strictures usually occur along the suture line.[1] From this viewpoint the procedure with more suture lines might have some negative effects on the long-term outcome. However, since vaginal stenosis at the suture line would be due to ischemia of the anastomotic site and undo tension,[1] we speculated that it could be avoided by tension-free anastomosis with the native vagina using well vascularized elastic tissue. Since severe stricture was uncommon in our study, advantages of the bilateral labioscrotal flap regarding its elasticity and vascularity seem to outweigh its disadvantages regarding its suture line.

Additionally, age at the operation is a critical issue in terms of the surgical outcome. Since endocrine stimuli, which occur in the first 2 months of life and after puberty, provide a larger, better vascularized vagina with thick walls that are easier to manipulate, surgery during this period is technically easier and recommended in the recent literature.[13–15] From the viewpoint of hormonal status surgery in the first 2 months of life would be recommended when feminizing genitoplasty is indicated early in life. However, in our study all except 1 patient underwent vaginoplasty between ages 2 to 6 years. Therefore, the perineal region and vaginal structures lacked the estrogenic influence at surgery in most of our cases, which could influence the long-term outcome.

There are some limitations in this study. 1) As described, some patients were slightly young for evaluation using the assessment system originally reported by Gupta et al.[10] 2) The number of patients was limited. 3) Since none of the patients achieved vaginal intercourse during followup, the patient reported outcome of our procedure in terms of the vaginal functional outcome for intercourse could not be assessed. To clarify the long-term outcome of this procedure at puberty or later in more patients further observations are necessary.

CONCLUSIONS

We report long-term followup in patients who underwent vaginoplasty with the bilateral labioscrotal flap. Although vaginal stenosis/stricture was observed at puberty in about half of the patients, severe stricture was uncommon. Serial evaluation for vaginal stenosis/stricture is recommended at the beginning of puberty for menstruation and several years later for vaginal intercourse in patients treated with vaginal reconstruction.

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EDITORIAL COMMENT

These authors used labioscrotal flaps with the hope that they would be more elastic and hormone responsive than posterior based perineal flaps. It is hard for me to conclude that to be the case since the reported results are well within the range of stenosis noted after using other flaps. Further comparison with such flaps would require careful cosmetic evaluation and the report of sexual function at intercourse. Since these patients were not yet sexually active, it would be premature to conclude that the finding of an adequate vagina on calibration ensures good function at intercourse or absent dyspareunia.

The most interesting finding was that 5 of 7 patients with stenosis at the onset of puberty had resolution without intervention. A potential explanation of the change could be that milder stenosis may improve through puberty with these types of flaps. I am not aware whether similar changes in circumference might or might not occur after other vaginoplasties during the same time frame. Another explanation could be that the criteria used to diagnose stenosis may have been too sensitive at the earlier developmental stage, creating false-positive findings. The changes suggest that we should be careful about evaluating results other than fistula or more severe stricture after vaginoplasty at a time frame short of the completion of puberty and particularly at the onset of sexual intercourse.

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REPLY BY AUTHORS

While various surgical techniques including traditional flap vaginoplasty have been reported, only a limited number have included long-term outcomes. Accordingly, final surgical outcome at puberty or later is still unknown for most of those techniques. We agree that it is hard to conclude that our procedure is apparently superior to other flaps and that this is an interim report of our long-term followup study. However, followup is ongoing for most of our patients and the final outcome of our procedure will be reported in the future.

Premature ovarian failure and androgen receptor gene CAG repeat lengths weighted by X chromosome inactivation patterns

The CAG repeat lengths weighted by X-inactivation ratios were significantly shorter in 58 Japanese patients with premature ovarian failure (POF) than in 42 age-matched control females with normal menses. The results suggest that short CAG repeats with a relatively high androgen receptor function may constitute a susceptibility factor for the development of POF. (Fertil Steril® 2009;91:649–52. ©2009 by American Society for Reproductive Medicine.)

Premature ovarian failure (POF) is a heterogeneous condition defined by the triad of primary or secondary amenorrhea, hypergonadotropism, and hypoestrinism in females less than 40 years old (1). While POF is frequently observed in females with sex chromosome aberrations, it also occurs in females with normal karyotypes (1). Although underlying factors for POF have been poorly elucidated in females with normal karyotypes, various genetic and environmental factors have been implicated in the development of POF. For example, mutations of several genes such as *BMP15*, *FOXL2*, and *NOBOX* as well as premutations of *FMR1* are known to cause POF (2–5), and several candidate genes such as *LHX8* and *GDF9* have been identified (6). Furthermore, chemotherapy, radiation, and autoimmune dysfunction also constitute risk factors for POF (1).

The androgen receptor (AR) plays a crucial role in sex development by mediating the biological effects of androgens (7). The *AR* gene resides on Xq12 and is made up of eight exons. Exon 1 harbors a highly polymorphic CAG repeat encoding a polyglutamine tract, and functional studies with different CAG repeat numbers have indicated an inverse relationship between the CAG repeat number and the transactivation function of *AR* (7). Consistent with this, the CAG repeat polymorphism is known to constitute a susceptibility factor for various androgen-related diseases in males (7). For example, while both positive and negative results have been reported, overall data from a large number of association studies argue that the CAG repeats tend to be long in males with undermasculinized genitalia and spermatogenic dysfunction and short in those with prostate cancers (7–9).

Similar association studies have also been performed in females with hirsutism and polycystic ovary syndrome (PCOS) together with X-inactivation analysis, revealing both positive and negative results (10–14). This would not necessarily be inconsistent with the CAG repeat polymorphism functioning as a susceptibility factor for androgen-related diseases in females as well as in males because the susceptibility effect may be detected in some patient groups but not in other patient groups. However, the data remain scanty, and further studies are necessary to draw a certain conclusion as to whether the CAG repeat polymorphism forms a susceptibility factor for androgen-related disorders in females. Thus, we performed CAG repeat length and X-inactivation analyses in POF patients because ovarian function is subject to androgen effects (1).

We studied 58 Japanese patients with POF. The menarcheal age ranged from 10 to 15 years (mean ± SD, 12.7 ± 1.2 years; menarcheal age in normal Japanese girls, 12.3 ± 1.3 years), and the age of POF onset (amenorrhea persisting ≥6 months) ranged from 13 to 39 years (median, 30 years). At the first medical examination, serum FSH was 44–245 IU/L (median, 94 IU/L), LH was 6–70 IU/L (median, 28 IU/L), and elevated FSH was repeatedly observed. Serum E_2 was undetectable in 45 patients and ranged from 10 to 72 pg/mL (35 to 250 pmol/L) in 13 patients. Serum T was not measured.

All 58 patients satisfied the following criteria: [1] lack of somatic abnormalities, [2] absence of clinically discernible autoimmune diseases, [3] no history of chemotherapy or radiation, [4] 46,XX karyotype in all the ≥30 lymphocytes examined, [5] no demonstrable mutations in the coding regions of *BMP15* and *GDF9*, and [6] no *FMR1* premutation. Two patients were familial cases with a similarly affected sister and/or mother, and the remaining 56 patients were sporadic cases. For controls, DNA samples from 42 Japanese females with proven fertility and normal menses aged 22–45 years (median, 34 years) were obtained from

Received September 12, 2007; revised and accepted November 28, 2007.
This study was supported by Grants for Child Health and Development (17C-2) and for Research on Children and Families (H18-005) from the Ministry of Health, Labor, and Welfare, and by Grants-in-Aid for Scientific Research (priority areas: 16086215; category B: 19390290) from the Ministry of Education, Culture, Sports, Science, and Technology, Japan.
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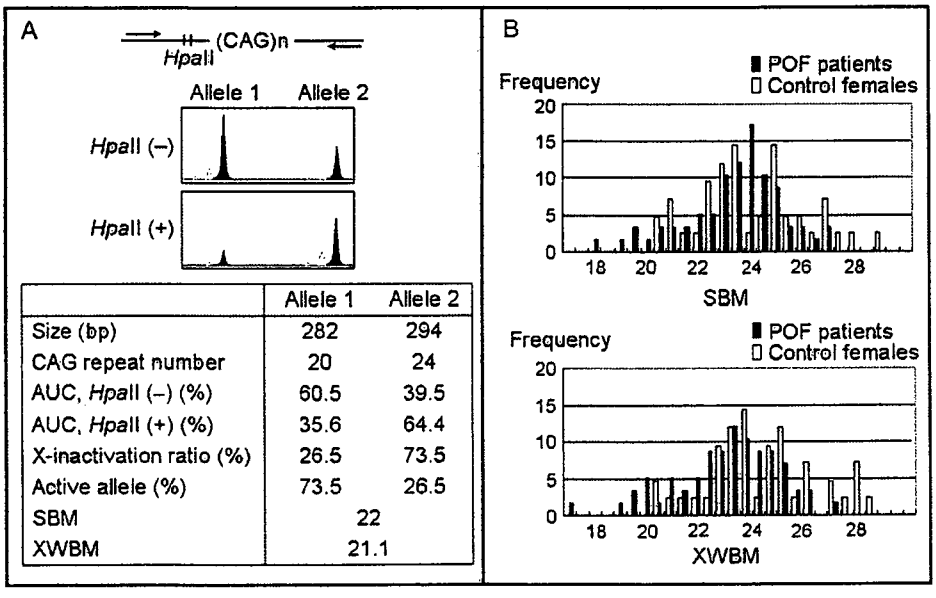
0015-0282/09/$36.00
doi:10.1016/j.fertnstert.2007.11.085

the Japanese Collection of Research Bioresources and similarly analyzed with permission. This study was approved by the Institutional Review Board committees of the investigators' affiliations. There is no conflict of interest.

CAG repeat length and X-inactivation analyses were performed by the previously reported method (15), with some modifications. In brief, leukocyte genomic DNA was polymerase chain reaction (PCR) amplified with a fluorescent labeled forward primer and an unlabeled reverse primer flanking the CAG repeat region and the two methylation sensitive *Hpa*II sites at exon 1 of *AR*, before and after *Hpa*II digestion (Fig. 1). The primer sequences and the PCR conditions were as described elsewhere (15). PCR products were obtained from both active and inactive X chromosomes before *Hpa*II digestion and from inactive X chromosomes alone after *Hpa*II digestion. For the CAG repeat length analysis, the PCR products obtained before *Hpa*II digestion were determined for size on an ABI PRISM 3100 autosequencer using GeneScan (Applied Biosystems, Norwalk, CT). Furthermore, to confirm the precise CAG repeat number, 12 PCR products of different sizes on GeneScan were subjected to direct sequencing on the autosequencer. For the X-inactivation analysis, the PCR products obtained before and after *Hpa*II digestion were examined for area under curve on the autosequencer. The X-inactivation ratio was calculated using the area under curve after compensation for unequal amplification of the two alleles caused by the difference in the product size. The CAG repeat number of each subject was obtained as the simple biallelic mean (SBM) and as the X-weighted biallelic mean (XWBM). The XWBM was calculated using the X-inactivation ratio and was expressed as a rounded number by increments of 0.5.

FIGURE 1

CAG repeat length and X-inactivation analyses. (**A**) Representative results. PCR amplification has been performed with a fluorescent labeled forward primer and an unlabeled reverse primer (arrows) flanking the CAG repeat region and the two methylation sensitive *Hpa*II sites at exon 1 of *AR*. Before *Hpa*II digestion, two alleles have been delineated on the autosequencer; allele 1 is 282 bp long and contains 20 CAG repeats, and allele 2 is 294 bp long and contains 24 CAG repeats. The difference in the area under curve (AUC) between the two alleles is primarily due to the short allele being more easily amplified than the long allele. The small 279 and 291 bp peaks are by-products caused by the slippage phenomenon. After *Hpa*II digestion, the two alleles have been detected, and the difference in the AUC pattern before and after the *Hpa*II digestion is primarily caused by noneven X-inactivation. The X-inactivation ratio, which is a mirror image of the active allele ratio, is calculated using the AUCs before and after *Hpa*II digestion. In this patient, the allele 2 is more preferentially inactivated than the allele 1, and the allele 1 and the allele 2 are expressed in 73.5% and 26.5% of leukocytes, respectively. Thus, the SBM is obtained as 22, and the XWBM is calculated as 21.1. (**B**) Distribution of the SBMs and the XWBMs in patients with POF and control females. The XWBM has been obtained as a rounded number by increments of 0.5; for example, calculated XWBM values from 22.75 to 23.24 have been rounded as 23, and those from 23.25 to 23.74 have been rounded as 23.5.



	Allele 1	Allele 2
Size (bp)	282	294
CAG repeat number	20	24
AUC, *Hpa*II (–) (%)	60.5	39.5
AUC, *Hpa*II (+) (%)	35.6	64.4
X-inactivation ratio (%)	26.5	73.5
Active allele (%)	73.5	26.5
SBM	22	
XWBM	21.1	

Sugawa. POF and AR CAG repeat polymorphism. Fertil Steril 2009.

Representative results and the distributions of the SBMs and the XWBMs are shown in Figure 1. The SBMs and the XWBMs were found to follow the normal distribution in both the POF patients and the control females by the χ^2-test, and the variances were shown to be similar between the two groups by the *F*-test. Thus, the Student's *t*-test was employed for the statistical analysis, showing that the SBMs were comparable between the POF patients and the control females (mean ± SD, 23.3 ± 2.0 vs. 24.1 ± 2.1; $P=.07$), whereas the XWBMs were mildly but significantly shorter in the POF patients than in the control females (mean ± SD, 23.2 ± 2.1 vs. 24.2 ± 2.2; $P=.02$). Neither the SBM nor the XWBM was found to be correlated with the menarcheal age ($r = -0.02$; $P=.90$), the age of POF onset ($r = 0.08$, $P=.58$), the serum FSH value ($r = 0.01$, $P=.94$), and the LH value ($r = -0.05$, $P=.78$) by the Spearman's ρ test.

The XWBM was mildly but significantly shorter in the patients with POF than in the control females, although the SBM was comparable between the two groups of subjects. In this context, while the AR function has not been compared between the two groups of subjects in this study, the previous studies have indicated an inverse relationship between the CAG repeat number and the AR function (7). Thus, a relatively high AR function in somatic cells may be a susceptibility factor for the development of POF because the AR function in somatic cells would be better reflected by the XWBM than by the SBM. Since *AR* is clearly expressed in the granulosa cells of developing follicles (16), increased AR function may affect the follicular cell function, facilitating the development of POF. Indeed, androgen excess in several conditions such as 21-hydroxylase deficiency and PCOS is known to impair ovarian function (1, 17), although there has been no report documenting the relationship between androgen excess and POF. One may argue that POF can also result from dysfunction of oocytes in which the AR function would simply be reflected by the SBM rather than the XWBM because the two X chromosomes remain active in oocytes (18). However, the relevance of an oocyte factor to POF is unlikely in terms of the AR function because *AR* is not expressed in oocytes (16).

The SBM and the XWBM were not correlated with the menarcheal and POF onset ages or the serum gonadotropin values. This would at least in part be due to variations in genetic and environmental factors influencing menarcheal and menopausal ages and hormonal values.

Several points should be made with respect to the present study. First, most of the control females were less than 40 years of age. This may have affected the results of this study because some of them may develop POF at a later age. Second, the X-inactivation pattern was examined for leukocytes in this study as well as in the previous studies of the CAG repeat polymorphism in females (10–14). Thus, although the X-inactivation ratio is similar among different tissues in most individuals (19), the XWBM may more or less be different between leukocytes and target tissues such as ovarian cells. Third, it remains to be examined whether CAG repeats tend to be short in other POF patients as well. Furthermore, POF may actually be associated with long CAG repeats with a relatively low AR function in ovarian follicular cells because POF is exhibited by female mice lacking *AR* (20). Thus, further studies are obviously necessary to examine the notion that short CAG repeats constitute a susceptibility factor for the development of POF.

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Clin Genet 2009: 75: 65–71
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CLINICAL GENETICS
doi: 10.1111/j.1399-0004.2008.01107.x

Short Report

CHD7 mutations in patients initially diagnosed with Kallmann syndrome – the clinical overlap with CHARGE syndrome

Jongmans MCJ, van Ravenswaaij-Arts CMA, Pitteloud N, Ogata T, Sato N, Claahsen-van der Grinten HL, van der Donk K, Seminara S, Bergman JEH, Brunner HG, Crowley Jr WF, Hoefsloot LH. *CHD7* mutations in patients initially diagnosed with Kallmann syndrome – the clinical overlap with CHARGE syndrome.
Clin Genet 2009: 75: 65–71. © Blackwell Munksgaard, 2008

Kallmann syndrome (KS) is the combination of hypogonadotropic hypogonadism and anosmia or hyposmia, two features that are also frequently present in CHARGE syndrome. CHARGE syndrome is caused by mutations in the *CHD7* gene. We performed analysis of *CHD7* in 36 patients with KS and 20 patients with normosmic idiopathic hypogonadotropic hypogonadism (nIHH) in whom mutations in *KAL1*, *FGFR1*, *PROK2* and *PROKR2* genes were excluded. Three of 56 KS/nIHH patients had *de novo* mutations in *CHD7*. In retrospect, these three *CHD7*-positive patients showed additional features that are seen in CHARGE syndrome. *CHD7* mutations can be present in KS patients who have additional features that are part of the CHARGE syndrome phenotype. We did not find mutations in patients with isolated KS. These findings imply that patients diagnosed with hypogonadotropic hypogonadism and anosmia should be screened for clinical features consistent with CHARGE syndrome. If such features are present, particularly deafness, dysmorphic ears and/or hypoplasia or aplasia of the semicircular canals, *CHD7* sequencing is recommended.

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Key words: anosmia – CHARGE syndrome – *CHD7* gene – hypogonadotropic hypogonadism – Kallmann syndrome

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Received 4 June 2008, revised and accepted for publication 27 August 2008

Kallmann syndrome (KS) is a congenital disorder that combines hypogonadotropic hypogonadism and anosmia (1). Three modes of inheritance have been described: X-linked recessive, autosomal dominant and more rarely autosomal recessive. To date, several genes have been identified to cause KS, either alone or in combination. Mutations in these genes together account for approximately 30% of all cases. *KAL1* encodes the protein anosmin and is involved in the X-linked

form of KS (KAL1, OMIM #308700) (2, 3). Loss-of-function mutations in the fibroblast growth factor receptor-1 gene (*FGFR1*) cause a form of KS (KAL2, OMIM #147950) that is generally inherited in an autosomal dominant way (4, 5). Dodé et al. reported in a further 10% of patients mutations in the prokineticin receptor-2 (*PROKR2*, KAL3, OMIM #607123) and prokineticin-2 (*PROK2*, KAL4, OMIM #607002) genes, encoding a cell surface receptor and one of its ligands, respectively (6). Mutations of the ligand, *PROK2*, can cause KS as well as normosmic idiopathic hypogonadotropic hypogonadism (nIHH) within the same family (6, 7). The same intrafamilial phenotypic variability is seen in patients with *FGFR1* mutations (4). Thus, KS is a phenotypically and genotypically heterogeneous disorder. Not only the degree of hypogonadism and anosmia may vary significantly but also other symptoms including bimanual synkinesia and dental agenesis (*KAL1* and *FGFR1*), renal anomalies (*KAL1*) and cleft lip/palate (*FGFR1*) occur with variable frequency (8).

CHARGE syndrome (OMIM #214800) is an autosomal dominant condition characterized by a variety of congenital anomalies including coloboma, heart defects, choanal atresia, retarded growth and development, genital hypoplasia, ear anomalies and deafness. Other commonly observed congenital defects are semicircular canal hypoplasia, facial nerve palsy, cleft lip/palate and tracheo-esophageal fistula (9). Our group has discovered *CHD7* as the causative gene in CHARGE syndrome (10). Since this discovery, several authors have reported on the phenotypic spectrum of *CHD7*-positive patients, including patients without typical CHARGE syndrome (11–13). Therefore, we presume that the mild end of the phenotypic spectrum of *CHD7* mutations is not yet completely explored.

Recent studies revealed that anosmia and abnormal olfactory bulb development, as well as hypogonadotropic hypogonadism, are almost consistent findings in CHARGE syndrome, indicating that the key features of KS are also present in CHARGE syndrome (14–16). For this reason, it has been suggested by others that *CHD7* may be considered a candidate locus in suspected KS cases without known mutations (8). This hypothesis is worthwhile exploring, also because mutations in *CHD7* can result in a much milder phenotype than the classical CHARGE syndrome phenotype. Therefore, we sequenced *CHD7* in a large group of patients diagnosed as KS or nIHH but without mutations in *KAL1*, *FGFR1*, *PROK2* and *PROKR2*.

Materials and methods

Patients

A cohort of seven Japanese patients with a clinical diagnosis of KS, without mutations in *KAL1*, *FGFR1*, *PROK2* and *PROKR2*, was screened for *CHD7* mutations (17). The diagnosis KS in this cohort was based on an underdevelopment of secondary sexual characteristics in combination with anosmia or hyposmia. Subsequently, the cohort was enlarged by 49 *KAL1*, *FGFR1*, *PROK2* and *PROKR2* negative North American patients with KS or nIHH. GnRH deficiency in this cohort was defined by (a) absent/incomplete puberty by age 18 year; (b) serum testosterone <100 ng/dl in men or estradiol <20 pg/ml in women in association with low or normal levels of serum gonadotropins; (c) otherwise normal pituitary function; (d) normal serum ferritin concentrations; and (e) normal magnetic resonance imaging (MRI) of the hypothalamic-pituitary region (5).

The patients in whom *CHD7* mutations were identified were carefully evaluated for clinical features of CHARGE syndrome. The *CHD7* gene was analyzed in the parents. The patients or their legal representatives gave informed consent for the DNA studies and the collection of clinical data. The studies were approved by the institutional review boards.

Mutation screening

DNA was isolated according to standard procedures. The 37 coding exons of the *CHD7* gene (exon 2–38, accession number NM_017780, NCBI) and their flanking intron sequences were amplified by polymerase chain reaction (PCR). Subsequently, sequence analysis was performed using a 3730 automated sequencer (Applied Biosystems, Foster City, CA). Primer information and PCR conditions are given in a previous report of our group (11).

The DNA samples of 11 mutation-negative patients were subsequently screened for exon deletions and/or duplications of the *CHD7* gene by multiplex ligation probe dependent amplification (MLPA) analysis (Table 1). We used a commercially available set of probes, the SALSA P201 kit (MRC-Holland, Amsterdam, The Netherlands; http://www.mrc-holland.com). Further details are described in our recent report on MLPA analysis of the *CHD7* gene (18).

Results

The *CHD7* gene was first screened in a cohort of seven *KAL1*, *FGFR1*, *PROK2* and *PROKR2*

Table 1. Clinical characteristics of all patients and results of *CHD7* analysis[a]

No.	Sex	Diagnosis	Additional features	Family	Mutation *CHD7*	Parents	MLPA performed
1	M	KS	Dental agenesis, high-arched palate, unilateral perceptive deafness and short stature	Sp	c.8803G>T; p.Glu2935X; exon 38	*De novo*	–
2	M	KS	Cleft palate, auricular dysplasia, nystagmus, bilateral perceptive deafness and hypoplasia of semicircular canals	Sp	c.6347T>A; p.Ile2116Asn; exon 31	*De novo*	–
3	F	KS		Sp	–		–
4	F	KS		Sp	–		–
5	M	KS	High-arched palate	Sp	–		–
6	M	KS	Ptosis	Sp	–		–
7	M	KS		Sp	–		–
8	F	KS	Facial nerve palsy, bilateral colobomas, cleft lip/palate, deafness, short stature and developmental delay	Sp	c.6070C>T; p.Arg2935X; exon 30	*De novo*	–
9	F	KS		Fam	–		+
10	M	KS		Fam	–		–
11	F	KS	Crohn's disease, syndactyly	Fam	–		–
12	M	KS		Fam	–		–
13	M	KS		Sp	–		+
14	F	KS		Sp	–		–
15	F	KS,	Choanal atresia	Fam	–		+
16	M	KS		Fam	–		–
17	M	KS	Congenital deafness and Hirschsprung's disease	Sp	–		–
18	F	KS		Fam	–		–
19	M	KS		Fam	–		–
20	F	KS		Fam	–		+
21	F	KS	Hearing impairment	Fam	–		–
22	M	KS	Deafness	Sp	–		–
23	F	KS	Multiple cranial nerve abnormalities	Sp	–		+
24	F	KS		Fam	–		–
25	F	KS		Sp	–		+
26	M	KS		Fam	–		–
27	M	KS	Hearing impairment	Sp	–		–
28	M	KS		Fam	–		–
29	M	KS		Fam	–		–
30	M	KS	Cryptorchidism	Fam	–		–
31	M	KS		Fam	–		–
32	F	KS	Narrow palate	Fam	–		–
33	F	KS	High-arched palate and hyperlaxity of hand joints	Fam	–		–
34	M	KS	Macrocephaly, hypertelorism, high-arched palate, ataxia, Dandy Walker malformation and developmental delay	U	–		–
35	M	KS		Fam	–		–
36	M	Partial KS	Spinal muscular atrophy	Sp	–		–
37	M	IHH, KS in family	Cardiac septum defect	Fam	–		+
38	M	IHH, KS in family	Hearing impairment	Fam	–		+
39	M	IHH, KS in family		Fam	–		–
40	F	IHH		Fam	–		+
41	M	IHH		Sp	–		+
42	F	IHH	Cardiac septum defect	Sp	–		+
43	M	IHH	Cryptorchidism	Fam	–		–
44	M	IHH	Growth hormone deficient	Fam	–		–
45	F	IHH		Fam	–		–
46	F	IHH		Fam	–		–
47	M	IHH	Cryptorchidism, blind, seizures, mental retardation and short stature	Sp	–		–
48	F	IHH		Fam	–		–
49	M	IHH	Ataxia	Sp	–		–
50	F	IHH		Fam	–		–
51	F	IHH		Fam	–		–

Table 1. Continued

No.	Sex	Diagnosis	Additional features	Family	Mutation *CHD7*	Parents	MLPA performed
52	M	IHH		Fam	–		–
53	M	IHH		Fam	–		–
54	M	IHH	Developmental delay and high-arched palate	Fam	–		–
55	M	IHH		Fam	–		–
56	F	IHH		Fam	–		–

F, female; Fam, familial; IHH, idiopathic hypogonadotropic hypogonadism; KS, Kallmann syndrome (IHH + anosmia); M, male; MLPA, multiplex ligation probe amplification; partial KS, patient with IHH and anosmia, with some degree of spontaneous pubertal development; Sp, sporadic; U, unknown.
[a]Patients 1–7 are of Japanese descent and patients 8–56 are from North America.

negative patients of Japanese descent (five males, two females). All had hypogonadotropic hypogonadism and anosmia, whereas some had additional symptoms. Their clinical features are summarized in Table 1, and patient 2 is shown in Fig. 1.

In two of the seven patients, a heterozygous mutation in *CHD7* was identified: one nonsense mutation (c.8803G>T; p.Glu2935X) and one missense mutation (c.6347T>A; p.Ile2116Asn). The mutations were proven to be *de novo* in both patients and were not present in 600 alleles of healthy controls.

The study cohort was extended by 49 North American patients (28 males, 21 females), including 29 patients with KS and 20 with nIHH of whom three had a positive family history for KS. Some of these patients had additional phenotypic features (Table 1). In one of the patients (patient 8), a *de novo* pathogenic nonsense mutation in *CHD7* was found (c.6070C>T; p.Arg2935X).

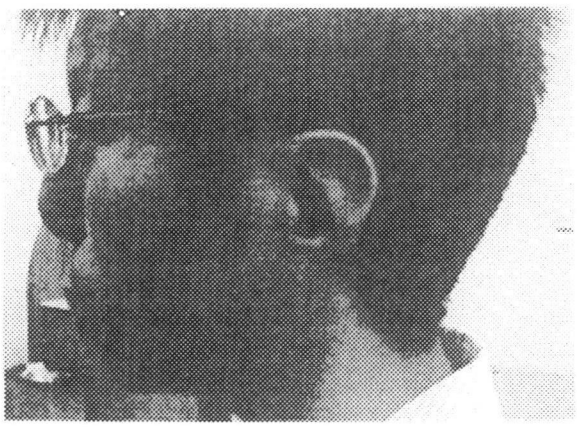

Fig. 1. Lateral view of patient 2. Note the dysmorphic ears with absence of the earlobe and the lower helical fold, and a triangular concha. These dysmorphism are typical for CHARGE syndrome.

As whole exon deletions or duplications will be missed by sequence analysis, we performed MLPA analysis. Due to a limited amount of available DNA, we were only able to finish this analysis in 11 patients. Two patients with a relatively high suspicion for CHARGE syndrome based on the features choanal atresia and multiple cranial nerve anomalies (respectively, patient 15 and 23; Table 1) were among those 11 patients. No exon copy number alterations were found.

The main features of the three patients carrying a mutation in *CHD7* are given in Table 1. All three patients were proven to be anosmic by formal smell tests. Audiometry revealed a left-sided hearing impairment of 70 dB in patient 1, a bilateral hearing impairment of 60–90 dB in patient 2 and left-sided complete sensorineural deafness and right-sided partial conductive hearing impairment in patient 8. Patient 1 had agenesis of four permanent teeth, the first upper and lower molars. No choanal atresia or heart defects were present in patients 1, 2 and 8. Colobomas were present in patient 8 but excluded by fundoscopy in patients 1 and 2. Patient 2 experienced feeding difficulties during infancy, but these were ascribed to the cleft palate. The dysmorphisms of the ears of patient 2 are very characteristic for CHARGE syndrome with absence of the earlobe and the lower helical fold, and a typical triangular concha (Fig. 1). After identification of the *CHD7* mutation, a CT scan of the os petrosum showed bilateral hypoplasia of the semicircular canals. In patients 1 and 8, imaging studies of the temporal bones were not possible. Upon re-evaluation, patient 8 has not only deafness and bilateral colobomas but also left-sided facial nerve palsy, cleft lip and palate, short stature and developmental delay.

In retrospect, patients 2 and 8 have typical CHARGE syndrome according to the commonly used clinical criteria (9), while patient 1 has only some features of this syndrome.