changes in the liver and hypertriglyceridemia developed, followed by a mild, abnormal pattern of subcutaneous fat distribution. An OGTT showed normal blood glucose responses, but with hyperinsulinism.

Methods and Results

Body composition analysis with dual-energy X-ray absorptiometry was performed in patient 1 using a Discovery® A Densitometer (Hologic, Inc., Bedford, MA, USA) in fan beam analysis mode and software version 13.3.0.1 (Fig. 1b). To illustrate fat distribution abnormality clearly, a 25-yr-old obese woman and a 29-yr-old healthy nonobese woman served as controls. In the former, this analysis was carried out as one of the routine medical evaluations for obesity.

Visceral fat area at the 4^{th} lumbar spine level was determined with FatVizCalc® (LISIT Co., Ltd., Tokyo, Japan) using the CT images of each patient (Fig. 1c).

Written informed consent for *LMNA* gene analysis was obtained from patients 2, 3 and 5 and from the mother of patient 1. Patient-specific gDNA was extracted from a nail specimen, and the common mutations in exons 8 and 11 of the *LMNA* gene, associated with FPLD2, were studied, as previously described (17). The mutation was absent in these patients (Table 2).

The onset of lipodystrophy is difficult to ascertain because the attending hematologists, as well as the patients themselves, are unaware of fat distribution abnormalities. Because serum triglyceride levels were routinely measured in the patients, we deduced the onset of lipodystrophy based on the timing of emergence of an elevated triglyceride level. As listed in Table 2, lipodystrophy seemed to develop about a decade after HSCT.

Discussion

All the described patients demonstrated a characteristic adipose tissue distribution pattern. Lipoatrophy was remarkable in the gluteal region and extremities, whereas subcutaneous fat was preserved, or even prominent, in the cheeks, neck and abdomen. Visceral fat deposition, as well as fatty changes in the livers of these patients, was also evident. This particular distribution pattern resembles that seen in FPLD2, which is caused by a *LMNA* gene mutation (1, 2, 5–7). Patients with this rare entity, which has an estimated prevalence of 1 in 15 million individuals (1), manifest a peculiar lipodystrophy after adolescence. In addition, metabolic complications, including insulin resistance, diabetes, hypertriglyceridemia, low HDL cholesterol levels and fatty liver, are prevalent in FPLD2 patients (3, 4). Female patients also have an increased risk for developing polycystic ovary syndrome and infertility (18). Compared with generalized lipodystrophy and other types of partial lipodystrophy, leptin and adiponectin levels in FPLD2 are only modestly decreased (19). However, FPLD2 is unlikely the cause of lipodystrophy in these patients, considering its low prevalence and the absence of *LMNA* gene mutations in the 4 patients tested.

Our patients and those with FPLD2 share similarities in fat distribution patterns and in metabolic derangements. Pronounced hypertriglyceridemia, coupled with decreased HDL cholesterol levels, was present in the 5 patients; elevated LDL cholesterol, defined as levels above 150 mg/dL, was present in 4 patients. The patients had high homeostasis model assessment ratios (HOMA-Rs), indicating insulin resistance, although acanthosis nigricans was not observed in any patients. Two patients had OGTTs that categorized them into the diabetic pattern according to the criteria developed by the Japanese Diabetes Society (see Fig. 2 legend). The patients with the diabetic pattern were found to have pronounced hyperinsulinemia with a peak insulin level exceeding 700 μIU/mL. In the present cases, the levels of leptin and adiponectin were modestly decreased.

The patients described in this report shared a common medical history that included HSCT

and conditioning with 10–12 Gy TBI. All of them also received intensive chemotherapy because of the severe nature of their diseases, including widespread metastases (patients 1, 4 and 5) and early relapses (patients 2, 3 and 4), and/or repetitive HSCT (patients 1, 3 and 5). Major surgery, however, was only conducted on those with neuroblastomas. Cranial radiation was performed on only 3 patients.

Based on the above observations, we propose HSCT as a new etiology for acquired partial lipodystrophy. Partial lipodystrophy seems to develop following HSCT, including TBI, especially in conjunction with intensive chemotherapy. This outcome appears to occur irrespective of other interventions such as surgery and cranial radiation. Younger age at the time of HSCT may be of significance, considering that 4 patients received transplants during infancy.

We speculate that TBI and/or intensive chemotherapy may damage the function of adipocytes in the subcutaneous fat, thereby limiting their lipid-storage capacity. This may lead to ectopic deposition of fat in visceral adipose tissue, muscle and liver. This hypothesis is reasonable because adipose tissue fibrosis, and the resultant ectopic lipid accumulation, has been demonstrated in obese individuals (20). Although the mechanism for the characteristic pattern of lipodystrophy is unclear, it may reflect the site-specific adipose tissue functions. In a subset of partial lipodystrophy accompanying glomerulonephritis, differential expression of complementary D by various adipose tissues is considered to cause the different degrees of lipodystrophy among the body (21).

It is essential to consider other potential factors that may be relevant to the development of lipodystrophy. Rooney and Ryan (22) reported a female patient who underwent allogeneic HSCT for relapsed ALL and developed partial lipodystrophy, with overt diabetes, 9 yr later. This patient had GVHD-associated scleroderma, and these authors speculated that there was a causative relationship between partial lipodystrophy and scleroderma. This hypothesis is very intriguing considering that decreased adiponectin levels have been described in systemic sclerodermas with autoimmune origins (23, 24). However, the severity of the GVHD varied among our patients, and scleroderma was present only in patient 3. GVHD was entirely absent in patient 4, who underwent autologous HSCT. Therefore, GVHD and GVHD-related scleroderma may not be a prerequisite but may be a predisposing factor for the development of partial lipodystrophy.

Another factor that should be considered is endocrinopathy. Four patients had endocrinological complications such as GHD, hypothyroidism and hypogonadism (Table 2). Although some of these endocrinopathies had been treated at the time of the investigation, hormone deficiency must be present for a significant period before the initiation of hormonal therapy. At present, endocrinopathy, *per se*, is not regarded as a definite cause of lipodystrophy (1, 2). However, endocrinological complications may be likely to modify the development and/or progress of lipodystrophy, considering that each hormone has its own receptor in the adipose tissue (25, 26), and the relationship between hormonal deficiency and metabolic complications is well-known (27, 28).

A causative relationship between HSCT and lipodystrophy may be disputed based on the absence of reports other than that of Rooney and Ryan (22). However, in accordance with our proposal, a high incidence of fatty liver was also reported in individuals who have undergone HSCT (29). In addition, radiation therapy, including TBI, is an established risk factor for developing metabolic syndrome (30, 31). Moreover, impaired glucose tolerance and dyslipidemia have been described as late complications following HSCT (32–34). We infer that a substantial number of partial lipodystrophy patients may have gone undiagnosed because careful observations are necessary to detect abnormal fat distribution

and because lipodystrophy is not a well-known condition, especially among pediatricians.

To clarify the incidence of HSCT-related lipodystrophy, as well as the contributions of GVHD, GVHD-scleroderma and endocrinopathies, further studies are clearly needed. Lipodystrophy appears to develop more than a decade after HSCT. In addition, the progress of lipodystrophy may be slow, considering that the OGTT results did not differ over a 10-yr interval in patient 3 (Table 3). Thus, prospective studies with long observation periods may be needed to clarify the reality of this potentially life-threatening complication in childhood cancer survivors.

Conclusion

Five pediatric patients manifesting aberrant fat distribution patterns similar to those observed in FPLD2 patients and severe metabolic abnormalities were described. Patients undergoing HSCT, especially when performed early in their lives and in conjunction with TBI and intensive chemotherapy, warrant careful observation for the potential development of partial lipodystrophy.

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CASE REPORT Open Access

Classic Bartter syndrome complicated with profound growth hormone deficiency: a case report

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Abstract

Introduction: Classic Bartter syndrome is a salt-wasting tubulopathy caused by mutations in the *CLCNKB* (chloride channel Kb) gene. Although growth hormone deficiency has been suggested as a cause for persistent growth failure in patients with classic Bartter syndrome, in our opinion the diagnoses of growth hormone deficiency has been unconvincing in some reports. Moreover, Gitelman syndrome seems to have been confused with Bartter syndrome in some cases in the literature. In the present work, we describe a new case with *CLCNKB* gene mutations and review the reported cases of classic Bartter syndrome associated with growth hormone deficiency.

Case presentation: Our patient was a Japanese boy diagnosed as having classic Bartter syndrome at eight months of age. The diagnosis of Bartter syndrome was confirmed by *CLCNKB* gene analysis, which revealed compound heterozygous mutations with deletion of exons 1 to 3 (derived from his mother) and ΔL130 (derived from his father). His medical therapy consisted of potassium (K), sodium chloride, spironolactone, and anti-inflammatory agents; this regime was started at eight months of age. Our patient was very short (131.1cm, -4.9 standard deviation) at 14.3 years and showed profoundly impaired growth hormone responses to pharmacological stimulants: 0.15μg/L to insulin-induced hypoglycemia and 0.39μg/L to arginine. His growth response to growth hormone therapy was excellent.

Conclusions: The present case strengthens the association between classic Bartter syndrome and growth hormone deficiency. We propose that growth hormone status should be considered while treating children with classic Bartter syndrome.

Keywords: Bartter syndrome, Salt-losing tubulopathy, Hypokalemia, Gitelman syndrome, Growth failure

Introduction

Classic Bartter syndrome (BS), also referred to as type III Bartter syndrome, is a rare genetic disorder characterized by salt wasting from the renal tubules, mainly the thick ascending loop of Henle [1]. It is caused by mutations in the *CLCNKB* gene that encodes the type b kidney chloride channel (ClC-Kb). Patients with classic BS fail to thrive from infancy and exhibit hypokalemia, metabolic alkalosis, hyperactive renin-aldosterone system, and overproduction of prostaglandins. Although potassium supplements, anti-aldosterone agents, and/or indomethacin are the mainstay of therapy, management of growth failure and hypokalemia is still challenging [1,2].

The association of growth hormone deficiency (GHD) with classic BS has been anecdotally reported, and GHD may be one of the causes of persistent growth failure frequently observed in patients with classic BS [2-8]. However, the degrees of GHD in the reported cases have been diverse, and hence, GHD has not yet been regarded as a definite complication of BS. In addition, most of the reported cases of BS accompanying GHD were not investigated on a molecular basis [3,7,8]. Moreover, Gitelman syndrome (GS) seems to have been confused with BS in older reports in the literature [4-6]. Here, we report a case of classic BS with documented *CLCNKB* gene mutations in a boy who was found to have profound

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GHD. We also present a literature review on the association between classic BS and GHD.

Case presentation

Our patient was a Japanese boy born at 41 weeks of gestation via spontaneous cephalic delivery, with a birth weight of 3,680g. His family history was remarkable in that his elder sister, who was five years older than him, had been diagnosed as having classic BS when she was five months old: her final height was 147.0cm (−2.1 standard deviation [SD]) and at a recent assessment her insulin-like growth factor 1 (IGF-1) level was 286ng/mL (normal range for her age, 168 to 459ng/mL).

At eight months of age, our patient was diagnosed as having classic BS based on the following findings: failure to thrive, metabolic alkalosis (pH 7.423; HCO_3^-, 33.6mmol/L; base excess, +8.2), hypokalemia (2.9mEq/L), and hyperactive renin-aldosterone system (plasma renin activity (PRA), 270ng/mL/h; normal value for his age, 2.58 ± 1.41ng/mL/h); aldosterone level, 850pg/mL (2,358pmol/L; normal value for his age, 173.7 ± 96.3pg/mL). The diagnosis of BS was confirmed by *CLCNKB* gene analysis, which revealed compound heterozygous mutations with deletion of exons 1 to 3 (derived from his mother) and ΔL130 (derived from his father), the latter of which has been reported previously by the authors TT and MA. Medical therapy consisting of potassium (K), sodium chloride, spironolactone, and anti-inflammatory agents was initiated at eight months of age and is still ongoing. However, as depicted in Figure 1, his serum K level remained considerably low because he was unable to consume large amounts of drugs, especially potassium preparations. Our patient also displayed mild intellectual impairment: he could only speak meaningful words by the age of three, and required specialized primary education.

Table 1 Results of pharmacological growth hormone stimulation tests in our patient at 14 years of age

	0 minutes	30 minutes	60 minutes	90 minutes	120 minutes
Insulin-induced hypoglycemia:					
Blood glucose (mg/dL)	94	54	93	99	92
Growth hormone (μg/L)	0.11	0.07	0.15	0.13	0.08
Arginine:					
Growth hormone (μg/L)	0.11	0.26	0.39	0.28	0.17

When he was 11 years old, an investigation for macrohematuria led to the detection of renal stones with nephrocalcinosis. This complication resolved following the amelioration of hypokalemia, which was achieved by our patient's increased efforts to ingest potassium tablets.

At 14.3 years of age, his severe short stature (131.1cm, -4.9SD) prompted us to evaluate his growth

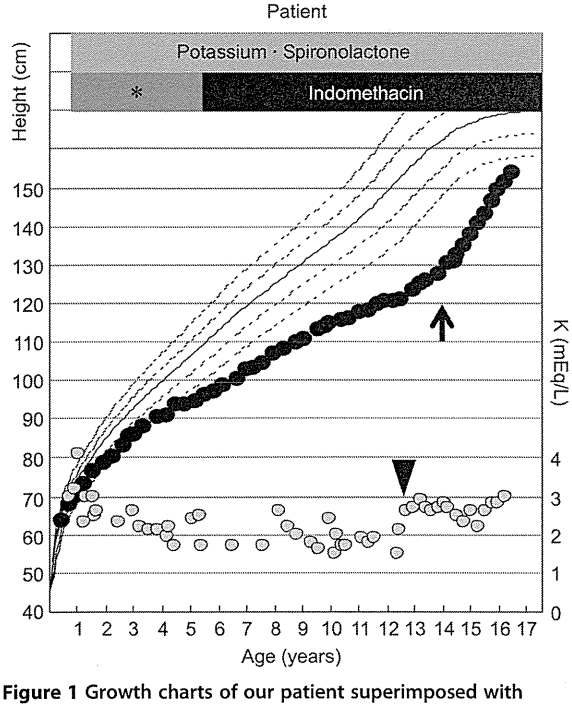

Figure 1 Growth charts of our patient superimposed with variations in his serum potassium levels. Black circles indicate heights; gray circles indicate potassium levels. The arrowhead indicates the age at which our patient could ingest potassium tablets, allowing higher potassium levels than before. Arrow indicates the initiation of growth hormone therapy. *Non-indomethacin anti-inflammatory agents such as tolmetin sodium and mefenamic acid.

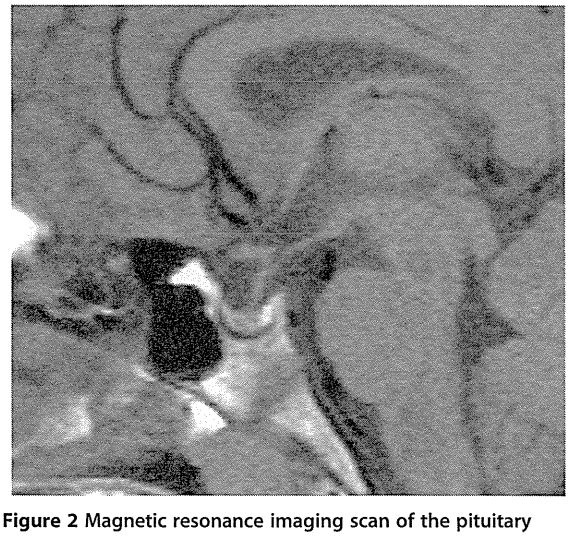

Figure 2 Magnetic resonance imaging scan of the pituitary gland of our patient.

Table 2 Classical Bartter syndrome with growth hormone deficiency: cases from the literature

Reference	Age, years	Sex	Mutation	GH peak (μg/L) to stimulants	IGF-1 (ng/mL)
[9]	5	M	IVS2-1G > C/W610X	9.3 (GLC), 8.0 (CLN), 8.2 (L-DOPA), 38.0 (ARG)	Not determined
[10]	8	F	Not determined	2.9 (INS), 2.0 (CLN), 6.9 (GRF)	122.1
[7]	10	M	Not determined	3.20 (INS), 3.20 (L-DOPA)	25
[8]	10	F	Not determined	0.70 (L-DOPA), 1.96 (CLN)	41.5
	11	M	Not determined	4.70 (L-DOPA), 1.79 (CLN)	39.7
	11	M	Not determined	0.50 (L-DOPA), 4.49 (CLN)	38.3
[2]	11	M	ΔExon1-6/ΔExon1-6	7.6 (ARG)	Low
	14	M	ΔExon1-19/ΔExon1-19	2.4 (ARG), 8.4 (GRF)	Low
[3]	22	F	Not determined	Absence (INS), 8.0 (ARG)	Not determined
Present case	14	M	ΔL130/ΔExon1-3	0.15 (INS), 0.39 (ARG)	80

ARG arginine, *CLN* clonidine, *L-DOPA* L-3,4-dihydroxyphenylalanine, *GH* growth hormone, *GLC* glucagon, *GRF*, growth hormone releasing factor, *IGF-1* insulin-like growth factor 1, *INS* insulin.

hormone (GH) status, and he was found to have profound GHD. His serum levels of IGF-1 and IGF binding protein 3 were 80ng/mL (normal range for his age, 178 to 686ng/mL) and 1.92μg/mL (normal range for his age, 2.69 to 4.16μg/mL), respectively. Pharmacologically stimulated GH levels were 0.15 and 0.39μg/L after insulin-induced hypoglycemia and arginine administration, respectively (Table 1). His bone age was 11.4 years (Tanner-Whitehouse 2-radius, ulna and short bones (TW2-RUS) method for Japanese individuals). Magnetic resonance imaging study results revealed no abnormalities in the hypothalamic-pituitary region (Figure 2).

GH therapy was initiated at 14.5 years of age at a dose of 21 to 27μg/kg/day, which restored his growth remarkably (Figure 1). Although his pubertal stage progressed from Tanner stage 1 to stage 2 over the next two years, his bone maturation (Δbone age/Δchronological age) was 1.02. No significant change was observed in his serum potassium level during GH therapy.

Discussion

To the best of our knowledge, the association of BS with GHD was first reported in 1977 [3]. Thereafter, a number of similar reports have been published [2-8]. However, we believe that some of the older cases reported in the literature do not comply with the current definition and concept of BS and thus should be recognized as GS [4-6]. GS is another salt-losing tubulopathy caused by mutations in the *SLC12A3* gene that encodes the thiazide-sensitive sodium-chloride cotransporter (NCCT) [1]. Because classic BS and GS shared the laboratory finding of hypokalemic alkalosis, these conditions were not strictly discriminated until the era of molecular diagnosis.

Table 3 Gitelman syndrome (including definite or probable cases) and GHD: cases from the literature

Reference	Age, years	Sex	Mutation	GH peak (μg/L) to stimulants	IGF-1
[12]	3	M	2614fr/unknown (*SLC12A3*)	<8 (INS), <8 (ARG), <8 (CLN)	Not determined
	9	F	G186D/unknown (*SLC12A3*)	6 (CLN)	89ng/mL
[5]	3	M	Not determined	3.3 (L-DOPA), 7.3 (CLN)	0.26U/mL
	9	F	Not determined	9.2 (L-DOPA), 4.8 (CLN)	0.67U/mL
	19	F	Not determined	6.0 (CLN)	Not determined
[6]	7	M	Not determined	9.8 (INS + ARG)	Not determined
[11]	9	M	Not determined	2.1 (INS), 3.2 (CLN), 1.8 (L-DOPA)	55ng/mL
[13]	10	F	Not determined	7.5 (L-DOPA), 6.9 (CLN)	Normal
[14]	11	M	Not determined	10.8 (GRF), 7.0 (CLN)	0.43U/mL
[15]	11	M	Not determined	5 (INS), 1 (CLN), 13 (GRF)	292ng/mL
[4]	11	M	Not determined	11 (CLN), 3.1 (GLC)	0.74U/mL
[16]	13	M	Not determined	5.4 (INS), 5.4 (ARG), 12 (GLC-PPL)	0.19U/mL

Cases were categorized as Gitelman syndrome according to the authors' own judgment, even if they were described as Bartter syndrome in the original reports.
ARG arginine, *CLN* clonidine, *L-DOPA* L-3,4-dihydroxyphenylalanine, *GH* growth hormone, *GLC* glucagon, *GRF* growth hormone releasing factor, *IGF-1* insulin-like growth factor 1, *INS* insulin, *PPL* propranolol.

Molecular diagnosis is a prerequisite for the detailed study of classic BS.

Tables 2 and 3 summarize cases of GHD reported to date classified as BS [2,3,7-10] and GS [4-6,11-16], respectively. Our patient's case is remarkable in that the diagnosis of classic BS was established molecularly. In addition, our patient's GH responses to pharmacological stimulants were most profoundly impaired among the hitherto reported cases. Although one may argue that hypokalemia may blunt the GH response and lead to false negative results, the excellent response to GH therapy made us suspicious for the presence of GHD. By adding our patient to the existing list of cases of GHD concomitant with BS, we believe that GHD should be regarded as a complication in classic BS.

Flyvbjerg *et al.* suggested that hypokalemia is a causative factor of GHD [17]. These authors stated that mice fed a low potassium diet showed growth retardation with low IGF-1 levels and attenuated GH response to GH-releasing factor (GRF). From this observation, hypokalemia seems to be one of the possible factors responsible for GHD in classic BS. This hypothesis is strengthened by the findings that GHD has also been reported in other diseases predisposing to hypokalemia, such as GS (Table 3) and the Bartter-like Dent disease [18]. In addition, this hypothesis can help to differentiate GHD (our patient in the present report) from non-GHD (his sister). Because large amounts of potassium could be administered via the gastric tube or tablets, a higher serum potassium level could be maintained in the sister, which may have prevented the development of GHD. Furthermore, the lack of association between GHD and antenatal BS, which is caused by mutations in either the *SLC12A1* (type I BS) or *KCNJ1* (type II BS) gene, can be explained by the observation that the correction of hypokalemia is generally easier in antenatal BS than in classic BS.

However, factors other than hypokalemia may be necessary for developing GHD. Patients with familial aldosteronism, rare genetic forms of primary aldosteronism, present with hypokalemia and some of them are refractory to medical therapy, yielding to long standing hypokalemia [19]. Regardless, GHD has not been reported to date in patients with familial aldosteronism. Thus, an aim of our future studies would be to determine the precise mechanism by which GHD develops in patients with classic BS.

Conclusions

In summary, we report our experience of profound GHD in a boy with mutations in the *CLCNKB* gene, and propose that GH status should be monitored while treating salt-losing tubulopathies including classic BS and GS.

Consent

Written informed consent was obtained from the patient's next-of-kin for publication of this case report and any accompanying images. A copy of the written consent is available for review by the Editor-in-Chief of this journal.

This study was approved by the Institutional Review Board of Kanagawa Children's Medical Center and followed the World Medical Association Declaration of Helsinki regarding ethical conduct of research involving human subjects.

Abbreviations

BS: Bartter syndrome; GH: Growth hormone; GHD: GH deficiency; GRF: GH-releasing factor; GS: Gitelman syndrome; IGF-1: Insulin-like growth factor 1.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

MA treated our patient from the beginning, performed the *CLCNKB* gene analysis and evaluated the GH status of our patient. MA also wrote the manuscript. TT and KM planned and performed the *CLCNKB* gene analysis. YA and KM critically reviewed and revised the manuscript. All authors read and approved the final manuscript.

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Novel Insights from Clinical Practice

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Characteristic Testicular Histology Is Useful for the Identification of NR5A1 Gene Mutations in Prepubertal 46,XY Patients

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Established Facts

- Leydig cells are not visible microscopically before puberty and their presence at this period is seen only in disorders of sexual development (DSD).
- The histological features of patients with *NR5A1* mutations have been described, but their clinical significance has not been fully established.

Novel Insights

- The key histological characteristics in the prepubertal testis of 46,XY patients with *NR5A1* mutations are hypoplastic seminiferous tubules and the emergence of Leydig cells with vacuolar cytoplasms.
- We propose that testicular histology is a useful marker for the identification of *NR5A1* mutations in 46,XY patients with DSD before puberty.

Key Words
Leydig cells · Steroidogenic factor-1 · Testicular histology · Steroidogenic acute regulatory protein

Abstract
Background: Individuals with *NR5A1* mutations encoding steroidogenic factor-1 (SF1) develop a phenotypically broad range of disorders of sexual development (DSD). Based on a literature review, we noted that hypoplastic seminiferous tubules and the emergence of Leydig cells with vacuolar cytoplasms are seen predominantly in the majority of individuals with *NR5A1* mutations. ***Aim:*** The aim of this study was to address whether the histopathological characteristics of the testis can be a biomarker for 46,XY individuals with *NR5A1* mutations. ***Design:*** In order to ascertain whether or not the histological features were the characteristics of *NR5A1* mutations, we screened the testicular histology of 242 patients

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with 46,XY DSD and then subsequently assessed *NR5A1* mutations. ***Result:*** Of 242 patients with 46,XY DSD, 6 patients matched histological testicular features: a reduced number of thin seminiferous tubules and focal aggregations of Leydig cells that contained cytoplasmic lipid droplets. All 6 patients had *NR5A1* mutations. These histological features were distinct from those of other DSD. Thus, this unique testicular histology is useful for identifying *NR5A1* mutations in 46,XY patients with DSD before puberty.

Introduction

The development and maturation of Leydig cells are dynamic processes involving interactions between hormones and differentiation of the male reproduction system [1]. Three distinct populations of Leydig cells have been described during testis development [2]. The fetal Leydig cell population undergoes the greatest increase in number during the second trimester, and they eventually become hyperplasic because of high levels of androgen hormone secretion [1]. Androgen hormone secretion from Leydig cells decreases following the virilization of the external genitalia and differentiation of the Wolffian duct, and the Leydig cells undergo involution after birth [3]. The infantile Leydig cell population appears a few months after birth and disappears shortly thereafter until puberty, during a period called minipuberty [4]. The adult Leydig cell population arises at puberty and maintains androgen production throughout adult life [1, 5]. It is hypothesized that Leydig cells adopt a fibroblastic phenotype and disappear from the postnatal time to puberty. Nevertheless, it is well established that Leydig cells are not histologically visible in the prepubertal testis.

Steroidogenic factor-1 (SF1; AD4BP; OMIM 184757), encoded by the *NR5A1* gene, is a nuclear receptor that regulates adrenal and reproductive development and function [6, 7]. Its role in mammalian development has been clarified using homozygous *nr5a1* knockout mice, which show a complete failure of adrenal and gonadal development, XY sex reversal, persistence of Müllerian structures in males, and impaired function of pituitary gonadotropes [8]. On immunohistochemical examination, expression of SF1 is initially observed in the sexually undifferentiated gonads of human embryos, then in the interstitial cells (the precursor of the Leydig cell) of the early testis, and then confined to Sertoli cells and Leydig cells later in testicular development [9]. These observations indicate that SF1 plays an important role in the differentiation, development, and function of Leydig cells.

The first human *NR5A1* mutation was found in a patient with 46,XY who had XY sex reversal and adrenal failure [10]. Since then, over 50 patients carrying *NR5A1* mutations have been reported [11–13]. The patients with *NR5A1* mutations had a broad range of external genitalia phenotypes that included normal-appearing female, ambiguous genitalia, hypospadias, undescended testes, anorchia, isolated micropenis, and normal male external genitalia with infertility [11–13]. The histological features of those patients have been described [10, 12, 14–21], but their clinical significance has not been fully established. We reviewed the literature and noted that some histological features were distinctive and were commonly seen in many patients with *NR5A1* mutations. Therefore, we examined our cohort to ascertain if the histological characteristics could be a surrogate maker for *NR5A1* mutations.

Patients and Methods

Literature Search

We reviewed the literature to search for clinical and histopathological features of patients with *NR5A1* mutations [10–12, 14–21]. Based on this review, we considered that there were three major histological types as follows (table 1): (1) streak gonads consisting of poorly formed seminiferous tubules in the connective tissue, (2) absence of the testicular tissue, and (3) testes composed of hypoplastic seminiferous tubules and/or the emergence of Leydig cells with occasional vacuolar cytoplasms. Both histological changes should be simultaneously found in a testis; however, descriptions concerning the presence of Leydig cells and the appearance of seminiferous tubules were occasionally incomplete. We hypothesized that hypoplastic seminiferous tubules and/or the presence of Leydig cells with vacuolar cytoplasms are the key pathological features of prepubertal 46,XY female patients with *NR5A1* mutations since either or both of these histological features are frequently described (table 1). We next investigated the relationship between these testicular histological features and the clinical subtype with *NR5A1* mutations. In order to determine the frequency of the histological characteristics in each clinical subtype, the patients with *NR5A1* mutations were classified as described by Achermann's group [11] with some modifications: type 1 = the typical 46,XY DSD phenotype characterized by ambiguous genitalia or clitorimegaly and absent or rudimentary Müllerian structures with undescended testes; type 2 = the severe 46,XY DSD phenotype represented by female external genitalia, a normal vagina, a normal uterus, and streak gonads; type 3 = hypospadias with small inguinal testes; type 4 = microphallus with or without bilateral anorchia, and type 5 = phenotypically normal men with nonobstructive male infertility (excluded from this study). In the current study, type 2 was defined as when the exter-

Table 1. Summary of previously published histological features of testes with *NR5A1* mutations

Patient No.	Age at gonadectomy	Seminiferous tubules	Germ cells	Leydig cells	Müllerian duct	*NR5A1* mutation	Ref.
1	4 months	abundant	many	ND	absent	V15M	[14]
2	4 months	decreased	present	ND	remnant	G91S	[14]
3	6 months	normal	normal	present	ND	c.536delC	[15]
4	6 months	normal	normal	ND	ND	c.536delC	[15]
5	7 months	decreased	sparse	vacuolated intersititial cell	remnant	M78I	[14]
6	15 months	immature	decreased	ND	absent	C16X	[16]
7	17 months	ND	decreased	marked	absent	R84C	[17]
8	18 months	ND	ND	hyperplasia	ND	W279X/G3314_3317delTCTC	[18]
9	2 years	ND	absent	aggregated Leydig cells	ND	Q206TfsX20	[12]
10	2 years	ND	absent	foamy Leydig cells	ND	L231_233dup	[12]
11	3.5 years	ND	ND	vacuolated Leydig cells	ND	H24T	[12]
12	4 years	ND	few	few	absent	C33S	[19]
13	4 years	ND	absent	few	absent	c.389delC	[12]
14	10 years	immature	ND	ND	present	G35E	[10]
15	10 months	immature	a few	absent	ND	R313H	[20]
16	6 years	atrophic	few	absent	ND	L437Q	[14]
17	4 years	atrophic	ND	ND	ND	V355M	[21]

Patient 14 was diagnosed as having streak gonads. In patient 17, the second biopsy at the age of 13 years showed an absence of testicular tissue. ND = Not described.

nal genitalia appeared to be complete female because information on the gonadal histology and the persistence of the Müllerian duct derivative was not always available. Patients with microphallus without anorchia were included in type 4. Type 5 was excluded because the patients in this category are normal-appearing males. Since Leydig cells are invisible until puberty and the presence of Leydig cells in prepuberty is a characteristic feature of *NR5A1* mutations, patients receiving gonadectomy at an age of 10 years or less were selected.

Patients and NR5A1 Mutation Analysis

In order to test whether or not the histological features proposed above could indeed predict the presence of *NR5A1* mutations, we screened for these features in 253 testes from 242 patients under 10 years of age with a 46,XY karyotype and abnormal sexual development who underwent gonadectomy or biopsy. These 242 patients were composed of 222 with unilateral or bilateral undescended testes, 5 with hypospadias with undescended testes, and 15 with ambiguous genitalia or complete female. To test whether other prepubertal patients with DSD lack the testicular histological features similar to patients with *NR5A1* mutations, we also histologically reviewed testes from patients with mixed gonadal dysgenesis (17 cases), androgen insufficiency (3 cases), Denys-Drash and Fraser syndromes (6 cases), and true hermaphroditism (4 cases) whose diagnoses were confirmed by cytogenetic or gene mutational analyses. Sequencing for *NR5A1* was performed on DNA from blood samples as previously described [22].

Written informed consent was obtained from the parents for the biochemical and molecular studies, which were approved by the ethical committee of Tokyo Metropolitan Children's Medical Center.

Pathological Examination

Testes that had the distinctive histological features were further examined to support the histological characteristics. Oil red O stain, immunohistochemistry, and electron microscopy were performed according to standard procedures. The following primary antibodies were used: SF1 (PMX, N1665, dilution 1:100) for the identification of Leydig cells and Sertoli cells, and WT1 (Nichirei, 6F-H2, diluted) and/or SOX9 (abcam, ab76997, dilution 1:100) for Sertoli cells. The size and number of the lumens of seminiferous tubules were quantified by morphometric analysis: the mean tubular diameter of each lumen and the number of tubules per unit area (0.64 mm^2) were measured using a digital image analyzer (WinRoof, Mitani Corp., Japan). After discrimination of Sertoli cells from spermatogonia by WT1 and/or SOX9 immunostaining, the number of spermatogonia per 10 tubular cross-sections and Sertoli cell number per tubular section were counted using the digital image analyzer. The resulting data were compared with previously published studies [23].

Results

Literature Review

The proportion of 46,XY DSD subtypes in 48 patients is shown in table 2, of which gonadal histology with the age of 10 years and less was available for 17 patients (tables 1, 3). Sixteen of 17 patients (94.2%) had hypoplastic seminiferous tubules and/or Leydig cells (table 3). Of

Table 2. The proportion of subtypes of patients with *NR5A1* mutations

Subtype	1	2	3	4	Summary
Number	30	7	9	2	48
Percent	62.5	14.6	18.7	4.2	100

Table 3. Relationship between histological type and clinical subtype in 46XY DSD with *NR5A1* mutations

	Type 1	Type 2	Type 3	Type 4	Summary
HST and/or Leydig cells	13[a]		2[b]	1	16
Streak		1			1
Anorchia					0

HST = Hypoplastic seminiferous tubules; Streak = streak gonads. All patients were under 10 years of age at gonadectomy.
[a] 1 case had no Leydig cells. [b] Both cases lacked Leydig cells.

these 16 cases, 13 (81.2%) belonged to the most common subtype (type 1; tables 2, 3), and the other 3 cases were types 3 and 4. Although there was a strong relationship between the proposed histological features and type 1 clinical phenotype, the histological features were also observed in other clinical subtypes, indicating that these histological features are common to patients with *NR5A1* mutations.

Identification of NR5A1 Mutations Based on Characteristic Histological Features

To test whether or not the two characteristic histological features could identify *NR5A1* mutations, we screened the histology of 253 testes from 242 patients with 46,XY. Among 253 testes, 6 patients had the matched histological characteristics (fig. 1a–h). As expected, all 6 patients had *NR5A1* mutations, all of which were novel mutations. Case 4 was recently reported [24]. The clinical, laboratory, and *NR5A1* mutation data for these 6 patients are summarized in table 4. Four of 6 cases fell into the category of the typical clinical phenotype (type 1), while 1 case had hypospadias with undescended testes (type 3). Although case 5 had completely female external genitalia, the testicular histology did not show streak gonads (fig. 1g, n). Thus, this patient might be classifiable as type 1 rather than type 2. The pathological significance of the identified mutations was confirmed by examining functional assays (unpubl. data). Remarkably, 3 patients (1–3 years of age) with undescended testes who received hCG load tests were included in this cohort, and none of them had *NR5A1* mutations. Thus, these cases eliminated the possibility that the hCG load test influenced the emergence of Leydig cells, and further supported the relationship between *NR5A1* mutations and the presence of Leydig cells. The characteristic histological features described in this study were not found in mixed gonadal dysgenesis, androgen insufficiency, Denys-Drash and Fraser syndromes, or true hermaphroditism (data not shown).

Detailed Pathological Evaluation for the Testes with NR5A1 Mutations

The pathological changes of the seminiferous tubules that underlie testicular hypoplasia and the emergence of Leydig cells with lipid accumulation were further examined by morphometric, immunohistochemical, and ultrastructural analyses. On gross examination, testicular hypoplasia was evident because the testicular parenchymas looked smaller as compared to the patients' epididymides (fig. 1a). Histologically, the testicular parenchyma consisted of a decreased number of seminiferous tubules. The seminiferous tubules were thin in younger patients (fig. 1b); however, the lumens of the seminiferous tubules tended to dilatate with age (fig. 1i, l), as is generally seen in testes with atrophy. The number of spermatogonia was decreased (fig. 1c, d, f–h, k–p). Sertoli cells had round or oval-shaped pyknotic nuclei and inconspicuous cytoplasms (fig. 1c, d, f–h). SF1, WT1, and SOX9 were expressed in the nuclei of the Sertoli cells (fig. 1k–p). The interstitium was often edematous, in which seminiferous tubules occasionally lacked back-to-back structures (fig. 1b). Case 3 was the oldest patient in our cohort whose testes were barely identifiable on microscopic examination. The seminiferous tubules were greatly reduced in number and showed atrophy and dilatation, suggesting that in addition to congenital hypoplasia, atrophic changes occurred secondarily. The second characteristic histological feature was the presence of focal aggregations of interstitial cells with foamy cytoplasms (fig. 1c–h), indicating the Leydig cells had intracytoplasmic accumulation of lipids. Nuclear immunostaining for SF1 confirmed that these interstitial cells were Leydig cells (fig. 1k–n). Lipid deposition in the cytoplasm was confirmed by oil red O staining (fig. 1i) and electron microscopic examination (fig. 1j). Müllerian duct remnants were not found in these 6 patients. The reduced size and number of sem-

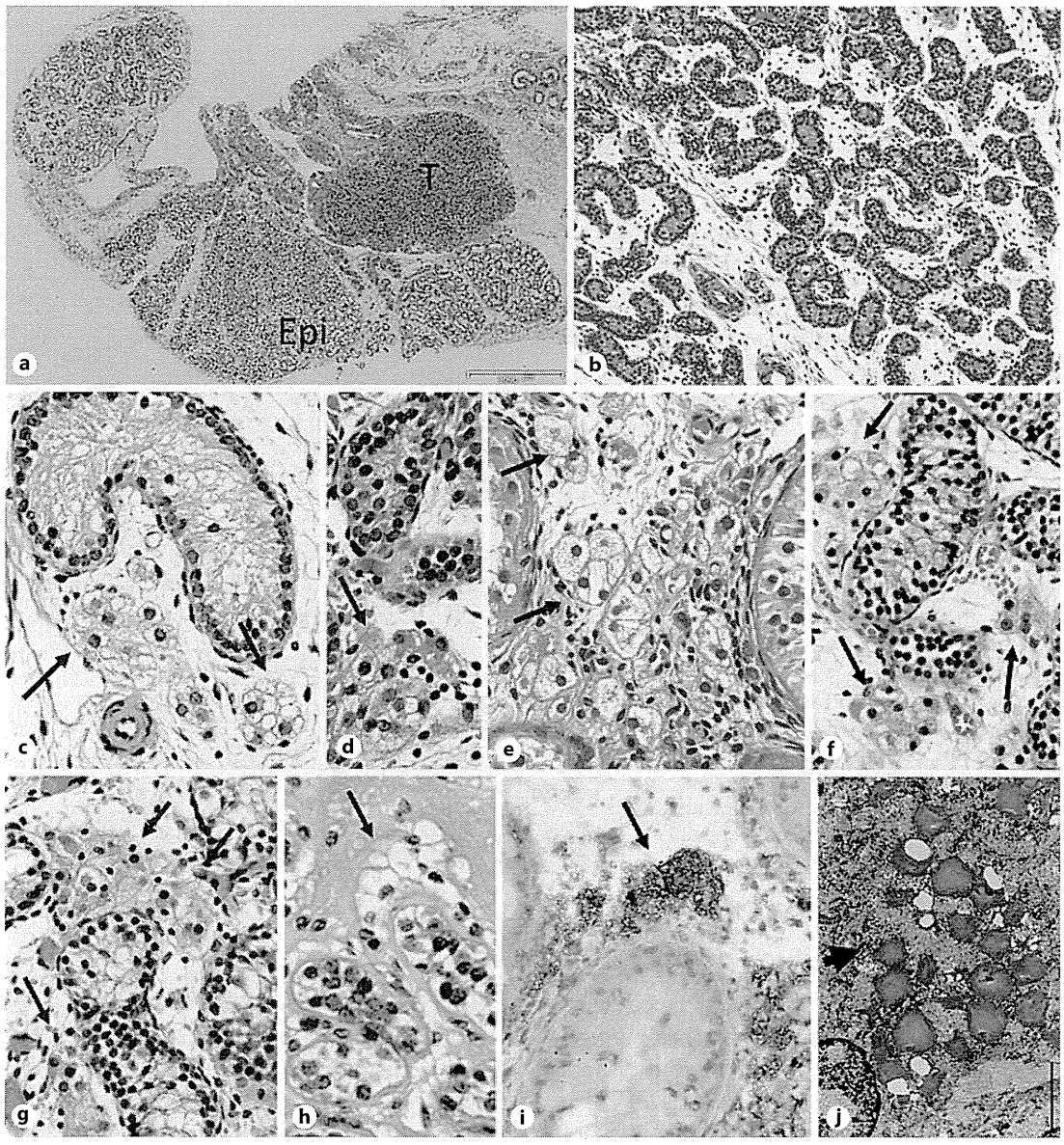

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Fig. 1. Pathological features of testes with *NR5A1* mutations. **a** Scanned image of a representative longitudinally bisected testis (case 1) showing a smaller testicular parenchyma (T) compared to the attached epididymis (Epi). **b** Microphotograph of a testicular parenchyma (case 2) revealing a decreased number of thin seminiferous tubules in the edematous loose connective tissue. High-power view of aggregated foamy Leydig cells (**c**, case 1; **d**, case 2; **e**, case 3; **f**, case 4; **g**, case 5; **h**, case 6; indicated by arrows). Note that spermatogonia are rarely seen in the seminiferous tubules. **i** Oil red O staining proving the accumulation of lipid droplets indicated by arrows (case 3). **j** Electron microscopy of Leydig cells (case 3) containing lipid droplets (high electron density deposits indicated by arrow heads).

iniferous tubules with a decreased number of spermatogonia underlying testicular hypoplasia were confirmed by the morphometric analysis (fig. 2a–c; table 5a, b), while the number of Sertoli cells was not significantly decreased.

Discussion

We confirmed the testicular histology associated with *NR5A1* mutations: hypoplastic testes are attributable to the reduced size and number of seminiferous tubules,

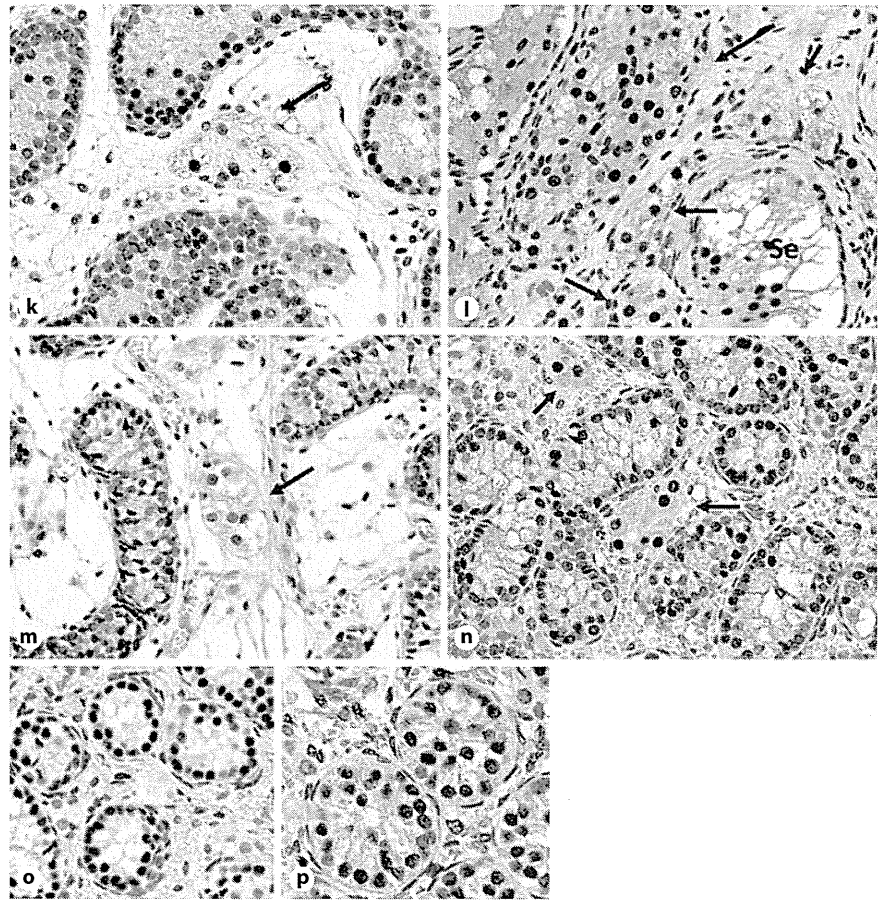

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Fig. 1. Pathological features of testes with *NR5A1* mutations. Immunohistochemistry for SF1 localizing in the nucleus confirming the emergence of Leydig cells indicated by arrows (**k**, case 1; **l**, case 3; **m**, case 4; **n**, case 5). SF1 is also expressed in Sertoli cells in the seminiferous tubules (Se). Sertoli cells have round or oval shaped nuclei expressing WT1 (**o**, case 5) and SOX9 (**p**, case 5) and inconspicuous cytoplasms. Original magnification ×200 (**b**), ×600 (**c–i**, **k–o**), ×400 (**p**).

which consist of round or oval-shaped Sertoli cells and few spermatogonia. Leydig cells are present with cytoplasmic lipid accumulations. We found that the histology is distinct from that of other DSD. The literature search demonstrated that these histological features are observed in the majority of patients with *NR5A1* mutations regardless of clinical subtype. Since the clinical features of types 1 and 2 46,XY DSD overlap considerably, type 2 therefore includes a number of patients who have the same histological features, such as we observed in case 5. It is thought that the pure form of patients with type 2 46,XY DSD essentially have streak gonads, but such patients are rare. Type 3 is the second most common subtype, whose testicular histology is rarely available because histopathological examination is seldom performed on undescended testes. We assume that type 3 patients also have the characteristic histological features because case 6 had identical histological features. An absence of Leydig cells was noted in 2 cases (table 1, patient Nos. 15 and 16). However, we could not confirm the absence of Leydig cells in these patients because the histologic evaluation was not performed on orchidectomy, but on biopsy samples which occasionally contain too little testicular parenchyma to reliably have any potential Leydig cells. Type 4 is also a rare subtype. Because anorchia (absence of the testicular tissue) results from secondary regressive changes, the testes originally might have also had the same histological characteristics. Type 5 was excluded from this study because the patients in this category are normal-appearing males. All types taken together, the characteristic histology is expected to be helpful for the identification of *NR5A1* mutations, but its utility would be limited for prepubertal patients with DSD.

It is conceivable that pathological changes in the testis with a *NR5A1* mutation primarily occur in Leydig cells and Sertoli cells because expression of SF1 is confined to these two cell types during testicular development. Dys-

Table 4. Clinical features and laboratory data of 6 patients with *NR5A1* mutations

Patient No.	Subtype	Mutation	Karyotype/ assigned gender	External genitalia	Müllerian duct	Age at gonadectomy	LH mIU/ml	FSH mIU/ml	Testosterone ng/ml[a]	Adrenal function
1	1	C13R	46,XY female	clitorimegaly	absent	1.6 years	basal ND peak 0.1	basal ND peak 6.5	basal <0.05 peak 0.20 (3 months)	normal
2	1	c.227_260del34	46,XY female	clitorimegaly	absent	6 months	basal 0.8 peak 7.9	basal 4.7 peak 4.8	basal 0.06 peak 0.61 (2 months)	normal
3	1	G26fsX5	46,XY female	clitorimegaly	absent	9.4 years	basal 2 peak 42.5	basal 37.6 peak 3.1	basal 0.17 peak 0.74 (9.4 years)	normal
4	1	D257TfsX39	46,XY female	clitorimegaly	absent	1.1 years	basal ND peak 3.2	basal ND peak 7.2	basal 0.92 peak 2.11 (1.1 years)	normal
5	2	P205fsX90	46,XY female	complete female	absent	4.3 years	basal 0.1 peak 10	basal 0.8 peak 7.9	basal 0.11 peak 1.33 (2.8 years)	normal
6	3	c.870+3_6delGAGT	46,XY male	hypospadia, bilateral cryptorchidism	absent	4 years	ND	ND	basal 0.17 peak 0.94 (9.4 years)	normal

[a] hCG stimulation (3,000 IU/m^2 intramuscularly daily for 3 days). ND = Not done.

function of Leydig cells is thought to be the main pathogenetic cause that explains the varying degrees of impaired sexual development of children with *NR5A1* mutations. Leydig cells are normally histologically invisible until puberty [3]; nevertheless, in patients with *NR5A1* mutations, Leydig cells are present with lipid accumulation. It can be speculated that accumulations of lipids in the Leydig cells disturb their ability to undergo the morphological change into a fibroblast-like phenotype, as normally occurs between the postnatal periods and puberty [3]. Therefore, the Leydig cells might be persistently identifiable. Alternatively, overstimulation of Leydig cells by human chorionic gonadotropin from syncytiotrophoblasts might cause emergence, hyperplasia, and persistence of Leydig cells beyond fetal stages. In a recent immunohistochemical study, Cools et al. [25] detected granular patterns of the SF1 protein in the cytoplasms of Leydig cells of 2 patients with *NR5A1* mutations, as well as those of adult Leydig cells. However, none of our 6 cases with *NR5A1* mutations showed a cytoplasmic granular SF1 staining pattern in Leydig cells, despite our use of the same SF1 antibody. The different SF1 immunostaining patterns between the two studies might be age related, as 2 patients in their study were 12 and 13 years of age, while all of our patients were under 10 years of age. On the other hand, Sertoli cells were thought to be functionally normal during the fetal period because of the absence of Müllerian structures and expression of WT1, SOX9, and SF1 in Sertoli cells without significant reduction in their number. Therefore, the morphological changes might have occurred secondarily.

Patients with *NR5A1* and steroidogenic acute regulatory protein *(StAR)* mutations share similar testicular histological features characterized by the presence of Leydig cells with lipid accumulation [26]. It is known that SF1 binds the *StAR* promoter and regulates its transcriptional activity leading to sex differentiation [27]. *NR5A1* mutations might cause accumulation of steroids in the Leydig cells through the same mechanism caused by *StAR* mutations. However, the mechanism remains to be elucidated. Interestingly, the histopathological features of the testes caused by the two mutations are largely different except for the presence of foamy Leydig cells. In *StAR* deficiency, seminiferous tubules develop nor-

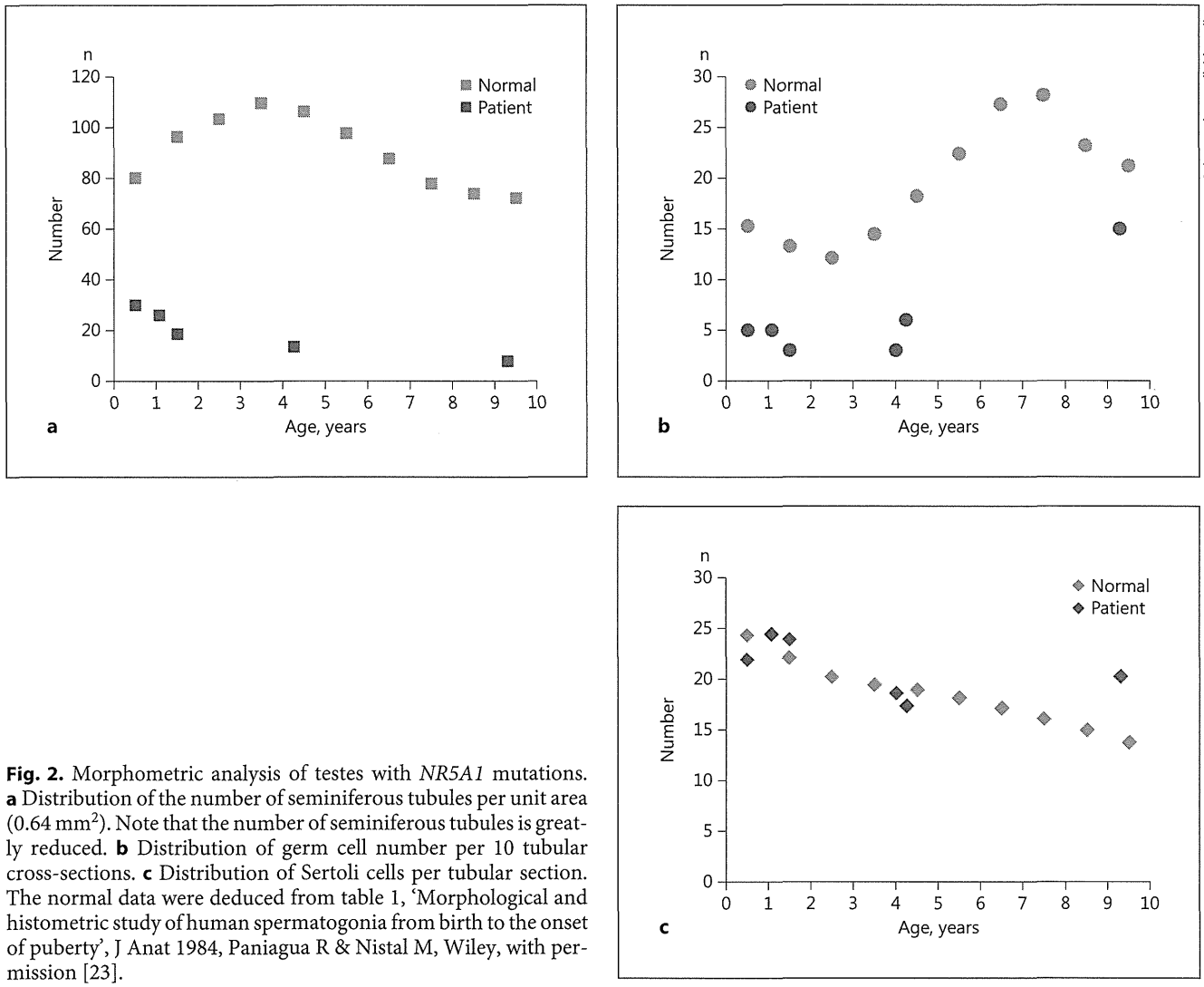

Fig. 2. Morphometric analysis of testes with *NR5A1* mutations. **a** Distribution of the number of seminiferous tubules per unit area (0.64 mm^2). Note that the number of seminiferous tubules is greatly reduced. **b** Distribution of germ cell number per 10 tubular cross-sections. **c** Distribution of Sertoli cells per tubular section. The normal data were deduced from table 1, 'Morphological and histometric study of human spermatogonia from birth to the onset of puberty', J Anat 1984, Paniagua R & Nistal M, Wiley, with permission [23].

mally, and germ cells in the seminiferous tubules are usually intact [28]. Thus, it is suggested that cells with *StAR* deficiency retain some capacity for androgen biosynthesis. In addition, the Sertoli cells can properly produce anti-Müllerian hormone, as the Müllerian duct completely regresses as usual. The size of the seminiferous tubules with *NR5A1* mutations is hypoplastic, while that of the testis with *StAR* mutations is normal at birth and becomes larger with more prominent lipid accumulation in Leydig cells with age [28]. Since *NR5A1* acts early in testicular development [9], it may regulate the size and the number of the seminiferous tubules, leading to a decreased number of Sertoli cells and germ cells. Leydig cells with *NR5A1* mutations do not become hypertrophic as compared to those with *StAR* mutations. This difference indicates that *StAR* might not be strictly regulated by SF1 in vivo.

Leydig cells can also sometimes be seen in other 46,XY DSD patients before puberty [29]. These include androgen dysfunction disorders (androgen insensitivity syndrome, 5α-reductase type II mutations) and mixed gonadal dysgenesis. As the testicular histology of androgen dysfunction disorders is similar to that of the cryptorchid testis, it might resemble that of *NR5A1* mutations [29]. However, neither congenital testicular hypoplasia nor foamy Leydig cells would be observed. The testicular histology of mixed gonadal dysgenesis is occasionally composed of abnormal architecture including areas of imma-

Table 5. Mean tubular diameter, number of tubules per unit area, and number of spermatogonia per 10 tubular cross-sections of prepubertal testes with normal (**a**) and *NR5A1* mutations (**b**)

a Normal control

	0–1 year	1–2 years	2–3 years	3–4 years	4–5 years	5–6 years	6–7 years	7–8 years	8–9 years	9–10 years
Mean tubular diameter, μm	84.1±2.0	77.4±2.1	74.5±1.9	72.1±1.8	70.0±1.3	71.9±1.5	74.4±1.5	77.5±1.4	80.2±1.7	83.9±1.9
Number of tubules per unit area (0.64 mm^2)	80.1±1.8	96.4±2.2	103.5±2.7	109.8±2.6	106.2±2.5	97.7±2.1	87.5±1.9	77.8±1.8	74.0±1.3	72.2±1.4
Number of spermatogonia per 10 tubular cross-sections	15.3±1.2	13.3±1.3	12.1±1.0	14.5±1.3	18.2±1.6	22.4±1.9	27.3±2.5	28.2±2.6	23.2±2.2	21.1±1.8
Sertoli cell number per tubular cross-section	24.4±0.3	22.2±0.3	20.3±0.3	19.5±0.3	19.0±0.3	18.2±0.3	17.2±0.3	16.1±0.2	15.0±0.2	13.8±0.2

Data are expressed as means ± SD per age group. Confidence limits are 99%. Data are deduced from table 1, 'Morphological and histometric study of human spermatogonia from birth to the onset of puberty', J Anat 1984, Paniagua R & Nistal M, Wiley, with permission [23].

b Patients with *NR5A1* mutations

	Patient 1 1.6 years	Patient 2 (lt) 6 months	Patient 3 (rt) 9.4 years	Patient 4 1.1 years	Patient 5 (rt) 4.3 years	Patient 6 (rt) 4 years
Mean tubular diameter, μm	22.5±0.7	30±3	8±1	26.1±1	13.7±3.4	52.5±0.7
Number of tubules per unit area (0.64 mm^2)	60.8±8.3	46.9±3.0	153.9±29.7	57.1±4.8	88.9±11.2	39.9±5.7
Number of spermatogonia per 10 tubular cross-sections	3	5	15	5	1	3
Sertoli cell number per tubular cross-section	24±2.6	22±2.5	20.3±5.1	24.5±1.7	17.4±2.0	18.7±4.0

Number of tubules per unit area, number of spermatogonia per 10 tubular cross-sections, and Sertoli cell number per tubular cross-section are plotted in fig. 2a–c, respectively.

ture primary sex cords indeterminate between female and male structures [29]. Thus, the histologic features are totally different from those of *NR5A1* mutations. In this study, we have ascertained that the other DSD patients with mixed gonadal dysgenesis, androgen insufficiency, Denys-Drash and Fraser syndromes, and true hermaphroditism lacked the testicular histological features similar to patients with *NR5A1* mutations.

In conclusion, we propose that testicular histological characteristics are a useful biomarker for the identification of *NR5A1* mutations in prepubertal 46,XY patients with DSD.

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