Novel Insights from Clinical Practice

HORMONE RESEARCH IN PÆDIATRICS

Horm Res Paediatr 2014;81:211–216
DOI: 10.1159/000356923

Received: June 24, 2013
Accepted: October 21, 2013
Published online: February 22, 2014

Tamoxifen Treatment for Pubertal Gynecomastia in Two Siblings with Partial Androgen Insensitivity Syndrome

Reiko Saito[a] Yukiyo Yamamoto[a] Motohide Goto[a] Shunsuke Araki[a]
Kazuyasu Kubo[a] Rinko Kawagoe[a] Yasusada Kawada[a] Koichi Kusuhara[a]
Maki Igarashi[b] Maki Fukami[b]

[a]Department of Pediatrics, School of Medicine, University of Occupational and Environmental Health, Kitakyushu, and [b]Department of Molecular Endocrinology, National Research Institute for Child Health and Development, Tokyo, Japan

Established Facts

- Most patients with partial androgen insensitivity syndrome (PAIS) manifest pubertal gynecomastia.
- Tamoxifen and other selective estrogen receptor modulators (SERMs) are fairly safe and effective for idiopathic pubertal gynecomastia.
- There have been no reports concerning the use of SERMs for patients with PAIS.

Novel Insights

- Tamoxifen is effective in treating pubertal gynecomastia in patients with PAIS.

Key Words
Androgen · Gynecomastia · Partial androgen insensitivity syndrome · Tamoxifen · Selective estrogen receptor modulator

Abstract
Background: Although tamoxifen has been shown to be fairly safe and effective for idiopathic pubertal gynecomastia, it remains unknown whether it is also beneficial for gynecomastia associated with endocrine disorders. Here, we report the effect of tamoxifen on pubertal gynecomastia in 2 siblings with partial androgen insensitivity syndrome (PAIS). ***Case Reports:*** Cases 1 and 2 presented with persistent pubertal gynecomastia at 13 and 16 years of age, respectively. Physical examinations revealed breast of Tanner stage 3 and normal male-type external genitalia in both cases. Clinical features such as female-type pubic hair and borderline small testis indicated mildly impaired masculinization. ***Results:*** Molecular analysis identified a previously reported p.Arg789Ser mutation in the androgen receptor gene *(AR)* in the 2 cases. Two months of oral administration of tamoxifen ameliorated gynecomastia to Tanner stage 2 with no adverse events. Additional treatment with testosterone enanthate showed negli-

KARGER

E-Mail karger@karger.com
www.karger.com/hrp

1663–2818/14/0813–0211$39.50/0

Yukiyo Yamamoto, MD, Department of Pediatrics
School of Medicine, University of Occupational and Environmental Health
1-1 Iseigaoka, Yahatanishi-ku
807-8555 Kitakyushu (Japan)
E-Mail y-yuki@med.uoeh-u.ac.jp

gible effects on body hair and penile length. Hormone values of the 2 cases during tamoxifen treatment remained similar to those in previously reported untreated patients with PAIS. ***Conclusion:*** The results indicate that tamoxifen was effective in treating pubertal gynecomastia in these 2 patients with PAIS and may be considered as a therapeutic option in this situation pending further studies.

Introduction

Pubertal gynecomastia is a benign condition primarily resulting from a hormonal imbalance between estrogens and androgens in breast tissue [1–5]. Gynecomastia of various degrees is seen in approximately 40–65% of adolescent boys [5]. Although pubertal gynecomastia is usually self-limited and spontaneously resolves within 1 year, this condition persists for several years in a small percentage of individuals [2, 6]. Pubertal gynecomastia is mostly idiopathic, although various chronic disorders, tumors, and drugs are known to cause this condition [1–5]. Notably, approximately 10% of cases of pubertal gynecomastia results from endocrine diseases such as aromatase excess syndrome, testicular dysfunction, and partial androgen insensitivity syndrome (PAIS) [2, 7].

Therapeutic interventions are required for pubertal gynecomastia associated with pain and/or psychological distress [2–4]. Previous studies have showed that tamoxifen and other selective estrogen receptor modulators (SERMs) are fairly safe and effective in treating idiopathic pubertal gynecomastia [2–4, 8, 9]. However, it remains unknown whether SERMs are also beneficial for pubertal gynecomastia resulting from endocrine disorders.

PAIS is an X-linked recessive disorder caused by hypomorphic mutations in the androgen receptor gene *(AR)* on Xq11-q12 [10, 11]. PAIS is associated with a broad phenotypic spectrum in genetic males, ranging from females with clitoromegaly to males with variable degrees of hypospadias, cryptorchidism, azoospermia, and pubertal gynecomastia [10–14]. Of these, pubertal gynecomastia appears to be the most common feature in patients with PAIS [12]. At present, however, there is no standard protocol for the management of gynecomastia in PAIS. Although surgery is usually recommended for this condition [10], surgical intervention has been associated with various minor complications such as skin retraction, hypertrophic scars, hypoesthesia, and skin redundancy [9]. Furthermore, while androgen supplementation therapy has been suggested to improve gynecomastia in PAIS [12], supporting evidence for this assumption is rather limited. In addition, no report has assessed the use of SERMs for gynecomastia in patients with PAIS. Here, we evaluated the effect of tamoxifen treatment on persistent pubertal gynecomastia in 2 siblings with PAIS.

Case Report

Case 1 was born at 40 weeks of gestation to nonconsanguineous Japanese parents. His postnatal growth and development were uneventful until puberty. He manifested gradually progressing bilateral breast enlargement from 12 years of age, and was referred to our clinic for the first time at 13 years of age. Physical examinations revealed breast of Tanner stage 3, axillary hair of stage 2, and female-type pubic hair of stage 3 (fig. 1). Bilateral testes were 8 ml in volume and palpable in the scrotum. The stretched penile length was 5 cm (–1.7 SD), and no hypospadias was noted. Clinical assessment excluded chronic disorders and medications that may cause gynecomastia [2]. The patient was therefore diagnosed as having idiopathic pubertal gynecomastia and treated with oral tamoxifen (20 mg/day). After 3 months he discontinued the treatment because the gynecomastia ameliorated to Tanner stage 2. At 14 years and 2 months of age, the patient returned to our clinic because of reappearance of gynecomastia.

Case 2 was the elder brother of case 1. He was born at 40 weeks of gestation and remained healthy throughout childhood. He developed bilateral gynecomastia at 13 years of age, and was referred to our clinic at 16 years of age when the breast enlargement became conspicuous. Physical examinations showed breast of Tanner stage 3, axillary hair of stage 2, and female-type pubic hair of stage 3 (fig. 1). Bilateral testes were palpable in the scrotum and 10 ml in volume. The stretched penile length was 6 cm (–1.8 SD). No hypospadias was noted. He had no signs of any chronic disorders or a history of drug use. The parents of the siblings were clinically normal.

Results

Molecular Analysis

This study was approved by the institutional review board committees at the National Center for Child Health and Development. After obtaining written informed consent, leukocyte genomic DNA samples were obtained from cases 1 and 2 and from their parents. G-banding analysis showed a normal 46,XY karyotype in cases 1 and 2. Direct sequence analysis for *AR* identified a hemizygous c.2367G>T, p.Arg789Ser mutation in both cases (fig. 2a, b). The p.Arg789Ser mutation was located in the ligand-binding domain of *AR*. In silico analysis using PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/) suggested p.Arg789Ser as a probable damaging mutation with a score of 0.999 (fig. 2b). The mother was heterozygous for this mutation.

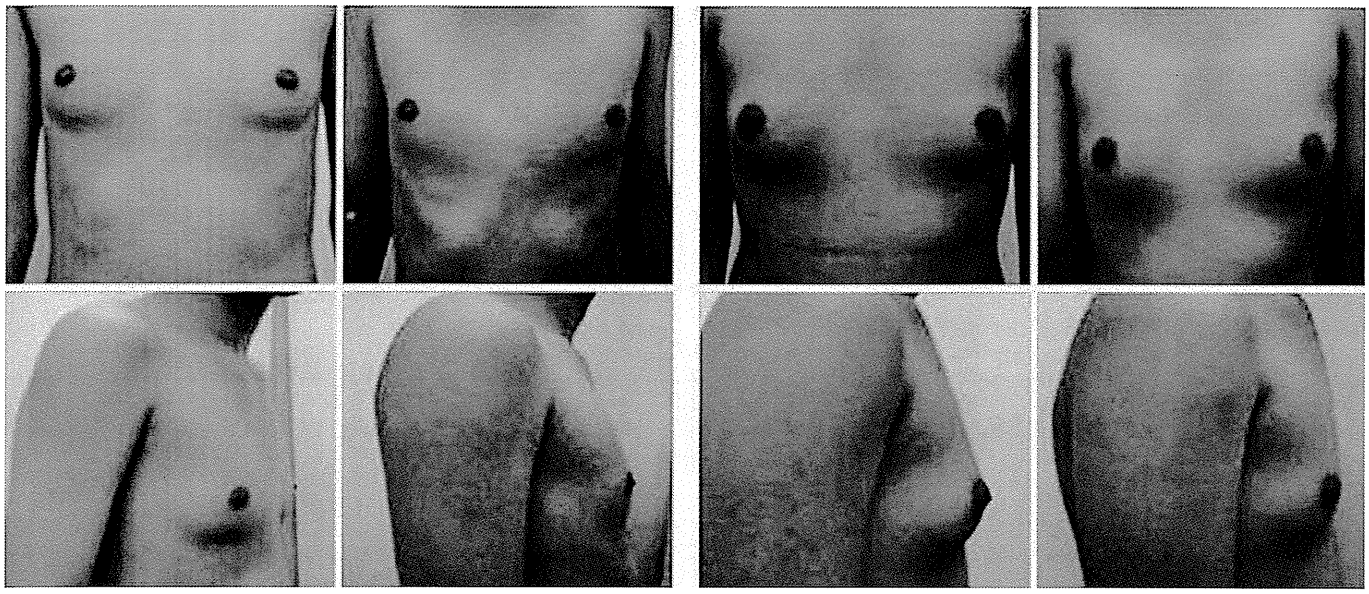

Fig. 1. Physical appearances of cases 1 (**a**) and 2 (**b**). Cases 1 and 2 presented with gynecomastia of Tanner stage 3. Tamoxifen treatment ameliorated gynecomastia to Tanner stage 2.

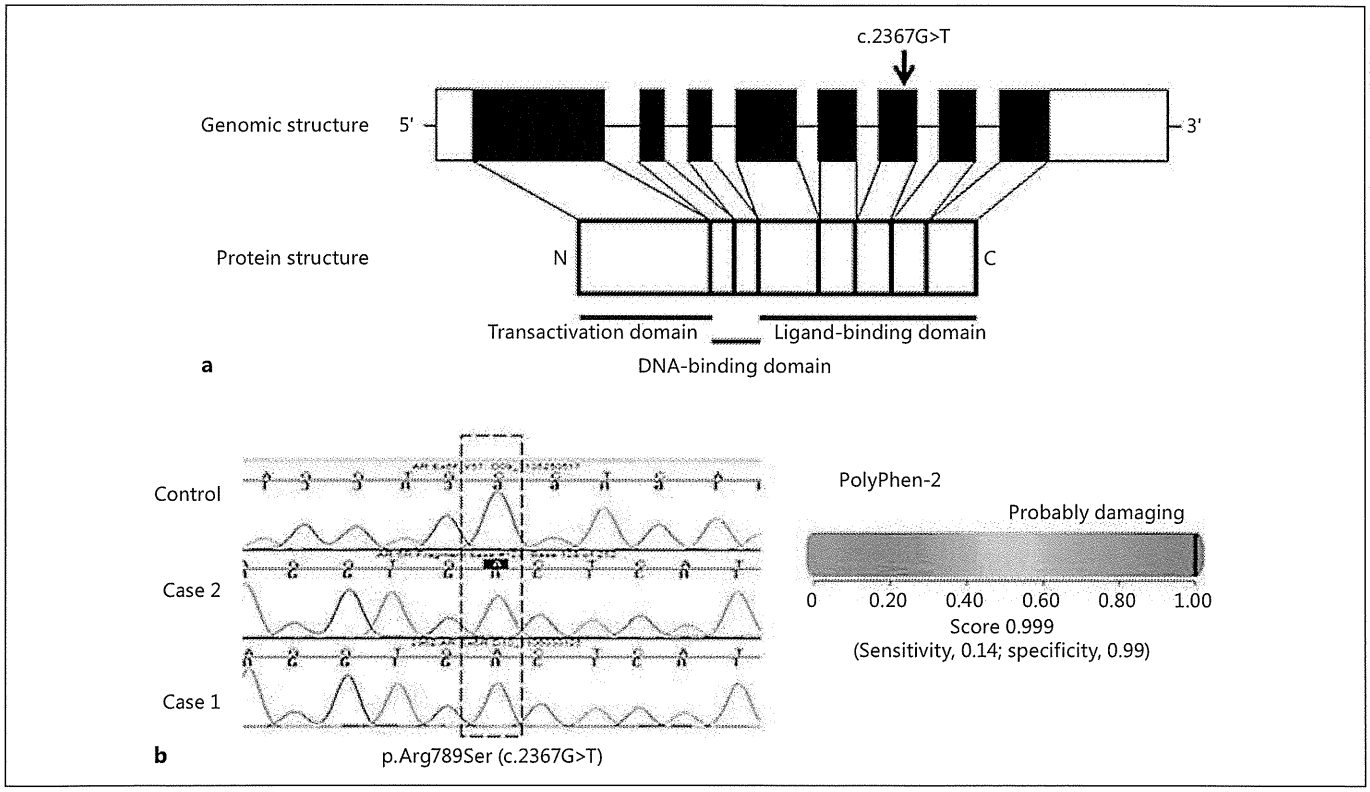

Fig. 2. Structure of the *AR* and the mutation identified in the present study. **a** Genomic and protein structures of *AR*. In the genomic structure, the white and black boxes indicate the noncoding and coding exons, respectively. The position of the nucleotide alteration (c.2367G>T) is indicated. **b** The results of direct sequencing (left panel, reverse sequences are shown) and in silico prediction analysis using PolyPhen-2 (right panel).

Table 1. Hormone values, clinical findings and treatment in cases 1 and 2

	Case 1					Case 2								Reference range[a]	
Age, years:months	13:00	13:03	14:02	14:04		15:09	16:07	16:00		16:02	16:03	16:05	16:10	17:07	
Hormone values	B	B	B	B	S[b]	B	B	B	S[b]			B	B	B	B	S[b]
LH, IU/l	3.7	**9.0**	5.1	**18.7**	**72.4**	**33.5**	**31.7**	5.5	20.1	...	...	**18.4**	**17.0**	**15.4**	2.9–8.2	18.2–38.0
FSH, IU/l	6.4	**12.2**	9.2	**20.2**	**26.8**	**13.9**	**12.3**	1.3	1.9	...	...	6.0	9.6	3.0	2.9–8.2	5.8–22.3
Testosterone, nmol/l	23.2	**51.0**	**47.5**	**114.4**		**69.7**	**74.5**	**36.8**		...	...	**46.8**	**49.9**	**53.7**	6.9–26.3	
Estradiol, pmol/l	<37	140	106	**419**		**345**	**275**	158		...	...	**389**	**297**	**294**	73–217	
TSH, mU/l	4.8		1.8					3.8							0.3–6.5	
free T4, pmol/l	11.6		16.7					14.2							11.6–21.9	
Prolactin, mU/l	578		417												152–557	
Clinical findings																
Breast (Tanner stage)	**3**	**2**	**3**	**2**		**2**	**2**	**3**		**3**	**2**	**2**	**2**	**2**		
Pubic hair (Tanner stage)	3			3		4	4	3					4	4		
Testis, ml	8			10		**10**	**10**	**10**					20	20		
Penile length (SD)	−0.7					**−3.0**	**−2.1**	−1.8					−1.8	**−3.3**		
Treatment																
Tamoxifen, mg/day	0→20[c]	20→0[c]	0→20[d]	20→40[e]		40	40			0→20[d]	20→40[e]	40	40	40		
TE, mg/month	...	...	...	...		0→150[f]	150			...	...	...	...	0→150[f]		

Hormone values below the reference range are underlined, and those above the reference range are boldfaced. Abnormal clinical findings are boldfaced. B = Basal; LH = luteinizing hormone; S = stimulated; TSH = thyroid-stimulating hormone. [a] Reference ranges for age-matched Japanese boys. [b] GnRH stimulation tests (100 μg bolus i.v.; blood sampling at 0, 30, 60, 90, and 120 min). [c] Tamoxifen was started at 13 years of age and discontinued after 3 months. [d] Tamoxifen was started at a dose of 20 mg/day. [e] Dose of tamoxifen was increased to 40 mg/day. [f] TE was started at a dose of 150 mg/month i.m.

Clinical Course

The clinical courses of cases 1 and 2 are shown in figure 1 and table 1. Since cases 1 and 2 experienced severe psychological distress due to gynecomastia, we initiated oral tamoxifen treatment (20 mg/day). Breast enlargement in both cases ameliorated from Tanner stage 3 to stage 2 after 2 months of tamoxifen treatment with no adverse events. After 3 months, the dose was increased to 40 mg/day. Since cases 1 and 2 were molecularly diagnosed with PAIS at 15.9 and 17.7 years of age, respectively, additional treatment with testosterone enanthate (TE; intramuscular injection, 150 mg/monthly) was started for correction of the mildly hypomasculinized genitalia and scarce body hair. TE treatment for 5 months induced laryngeal prominence and acne in the 2 cases; however, it caused no remarkable changes in penile length or body hair.

Hormonal Findings

The endocrine findings of cases 1 and 2 are summarized in table 1. Before tamoxifen therapy, their blood hormone levels were within the normal range, except for decreased estradiol levels in case 1, and decreased follicle-stimulating hormone (FSH) and slightly increased testosterone levels in case 2. During tamoxifen treatment, the blood levels of luteinizing hormone, testosterone, and estradiol were markedly elevated in both cases. In addition, the basal and GnRH-stimulated levels of FSH during treatment were significantly elevated in case 1, while they remained within the normal range in case 2. Additional treatment with TE caused no remarkable changes in the hormone values of case 1. Endocrine data for case 2 under TE treatment were not available.

Discussion

We identified a p.Arg789Ser mutation in *AR* in 2 siblings with persistent pubertal gynecomastia and various symptoms of mild hypomasculinization. The phenotypes of cases 1 and 2 could be ascribed to this mutation because in silico analysis suggested p.Arg789Ser as a probable damaging mutation. In fact, p.Arg789Ser has previously been detected in a patient with gynecomastia, a high-pitched voice, and decreased body hair [15], and in a patient with gynecomastia and ambiguous genitalia [16]. These findings support the notion that persistent pubertal gynecomastia is an essential feature in patients with hypomorphic mutations in *AR* [12].

Tamoxifen treatment for 2 months significantly ameliorated gynecomastia in the 2 cases. These results indicate that in the breast tissue, the antiestrogenic effect of tamoxifen can counterbalance the impaired androgen action caused by *AR* mutations. In this regard, it is worth mentioning that SERMs have been used for a number of patients with idiopathic pubertal gynecomastia [3, 8, 9]. Although other therapeutic options, such as aromatase inhibitor therapy and surgery have also been used for idiopathic pubertal gynecomastia, the response rate to therapy with SERMs (84–90%) is significantly higher than that to aromatase inhibitor therapy (almost equivalent to placebo) [2, 4, 8]. Previous case series have reported success with tamoxifen at a dosage of 20–40 mg daily for 2–9 months for idiopathic pubertal gynecomastia and few recurrences of gynecomastia after cessation of therapy [2, 4]; however, the tamoxifen dose and duration for the PAIS patients is unclear. Future studies are necessary to clarify the recommended dose and duration of tamoxifen treatment for PAIS patients.

When gynecomastia is present for more than 1 year, the tissue becomes inactive and fibrotic [2]. In our 2 cases, tamoxifen treatment showed a significant effect in amelioration of gynecomastia within 2 months; however, mild gynecomastia still remained. This may be caused by progress to dense fibrosis. Furthermore, while SERMs are rarely associated with adverse events in adolescent boys [8, 9], surgery is known to result in various minor complications in more than 50% of patients [9]. Therefore, SERMs are considered to be useful for idiopathic pubertal gynecomastia.

The present study shows for the first time that tamoxifen can also ameliorate pubertal gynecomastia associated with PAIS. Our results, when considered together with significantly improved spermatogenesis after tamoxifen treatment in an adult patient with PAIS [17], imply that SERMs may provide considerable benefit to patients with PAIS. In this context, androgen supplementation therapy may be considered to be more helpful than SERMs for patients with PAIS because androgens appear to improve not only gynecomastia but also genital abnormalities [11, 12]. However, the effect of androgens may vary among patients depending on the functional activities of the mutant AR proteins [10, 11]. Indeed, TE treatment induced only minor responses in our cases. Thus, further studies are necessary to clarify the effects of SERMs and androgens in patients with PAIS.

The endocrine evaluation of cases 1 and 2 provided notable findings. Both cases had almost normal hormone values before tamoxifen treatment, and significantly increased levels of luteinizing hormone, testosterone and estradiol during treatment. These hormone data are almost comparable to those in previously reported untreated patients with PAIS [12]. Furthermore, markedly elevated FSH levels similar to those in case 1 have been reported in several patients with PAIS [12]. Such hormone alterations can be explained by perturbed feedback regulation of gonadotropins resulting from impaired AR function in the brain [10, 11]. However, since tamoxifen is known to exert a stimulatory effect on the hypothalamus-pituitary-gonadal axis in normal males [18], it remains possible that tamoxifen played a role in the hormone abnormalities of our cases.

In summary, our findings indicate that tamoxifen is effective in treating pubertal gynecomastia in patients with PAIS. These results provide a novel therapeutic option for gynecomastia associated with endocrine disorders. Further studies that compare the effects of SERMs and androgens, along with longitudinal monitoring for hormone levels, are necessary to establish an optimal management protocol for gynecomastia in patients with PAIS.

Acknowledgements

This work was supported by a Grant-in-Aid for Scientific Research on Innovative Areas (22132004) from the Ministry of Education, Culture, Sports, Science and Technology; by a Grant for Research on Intractable Diseases from the Ministry of Health, Labor and Welfare, and by a Grant from National Center for Child Health and Development (23A-1).

Disclosure Statement

The authors have nothing to disclose.

References

1 Ma NS, Geffner ME: Gynecomastia in prepubertal and pubertal men. Curr Opin Pediatr 2008;20:465–470.
2 Braunstein GD: Gynecomastia. N Engl J Med 2007;357:1229–1237.
3 Johnson RE, Murad MH: Gynecomastia: pathophysiology, evaluation, and management. Mayo Clin Proc 2009;84:1010–1015.
4 Barros AC, Sampaoio Mde C: Gynecomastia: physiopathology, evaluation and treatment. Sao Paulo Med J 2012;130:187–197.
5 Nordt CA, DiVasta AD: Gynecomastia in adolescents. Curr Opin Pediatr 2008;20:375–382.

6 Biro FM, Lucky AW, Huster GA, Morrison JA: Hormonal studies and physical maturation in adolescent gynecomastia. J Pediatr 1990;116:450–455.
7 Sher ES, Migeon CJ, Berkovitz GD: Evaluation of boys with marked breast development at puberty. Clin Pediatr 1998;37:367–372.
8 Maidment SL: Question 2. Which medications effectively reduce pubertal gynecomastia? Arch Dis Child 2010;95:237–239.
9 Lawrence SE, Faught KA, Vethamuthu J, Lawson ML: Beneficial effects of raloxifene and tamoxifen in the treatment of pubertal gynecomastia. J Pediatr 2004;145:71–76.
10 Hughes IA, Davies JD, Bunch TI, Pasterski V, Mastroyannopoulou K, MacDougall J: Androgen insensitivity syndrome. Lancet 2012; 12:1–10.
11 Achermann JC, Hughes IA: Disorders of sex development; in Melmed S, Polonsky KS, Larson PR, Kronenberg HM (eds): Williams Textbook of Endocrinology, ed 12. Philadelphia, Saunders, 2011, pp 868–934.
12 Hellmann P, Christiansen P, Johannsen TH, Main KM, Duno M, Juul A: Male patients with partial androgen insensitivity syndrome: a longitudinal follow-up of growth, reproductive hormones and the development of gynecomastia. Arch Dis Child 2012;97:403–409.
13 Audi L, Fernández-Cancio M, Carrascosa A, Andaluz P, Torán N, Piró C, Vilaró E, Vicens-Calvet E, Gussinyé M, Albisu MA, Yeste D, Clemente M, Hernández de la Calle I, Del Campo M, Vendrell T, Blanco A, Martínez-Mora J, Granada ML, Salinas I, Forn J, Calaf J, Angerri O, Martínez-Sopena MJ, Del Valle J, García E, Gracia-Bouthelier R, Lapunzina P, Mayayo E, Labarta JI, Lledó G, Sánchez Del Pozo J, Arroyo J, Pérez-Aytes A, Beneyto M, Segura A, Borrás V, Gabau E, Caimarí M, Rodríguez A, Martínez-Aedo MJ, Carrera M, Castaño L, Andrade M, Bermúdez de la Vega JA; Grupo de Apoyo al Síndrome de Insensibilidad a los Andrógenos (GrApSIA): Novel (60%) and recurrent (40%) androgen receptor gene mutations in a series of 59 patients with a 46,XY disorder of sex development. J Clin Endocrinol Metab 2010;95:1876–1888.
14 Ahmed SF, Cheng A, Dovey L, Hawkins JR, Martin H, Rowland J, Shimura N, Tait AD, Hughes IA: Phenotypic features, androgen receptor binding, and mutation analysis in 278 clinical cases reported as androgen insensitivity syndrome. J Clin Endocrinol Metab 2000; 85:658–665.
15 Deeb A, Mason C, Lee YS, Hughes A: Correlation between genotype-phenotype and sex of rearing in 111 patients with partial androgen insensitivity syndrome. Clin Endocrinol 2005;63:56–62.
16 Lumbroso R, McPhaul MJ, Trifiro MA, Pinsky L: A region of the human androgen receptor that in a mutational hot spot for distinct ligand kinetics and mild androgen resistance. Endocrine Society 81st Annual Meeting, Durham, 1999, p 500.
17 Gooren L: Improvement of spermatogenesis after treatment with the antiestrogen tamoxifen in a man with the incomplete androgen insensitivity syndrome. J Clin Endocrinol Metab 1989;68:1207–1210.
18 Vermeulen A, Comhaire F: Hormonal effects of an antiestrogen, tamoxifen, in normal and oligospermic men. Fertil Steril 1978;29:320–327.

Clin Pediatr Endocrinol 2014; 23(3), 65–72

Review

Skeletal Deformity Associated with *SHOX* Deficiency

Atsuhito Seki[1], Tomoko Jinno[2], Erina Suzuki[2], Shinichiro Takayama[1], Tsutomu Ogata[3], and Maki Fukami[2]

[1]*Department of Orthopedic Surgery, National Center for Child Health and Development, Tokyo, Japan*

[2]*Department of Molecular Endocrinology, National Research Institute for Child Health and Development, Tokyo, Japan*

[3]*Department of Pediatrics, Hamamatsu University School of Medicine, Hamamatsu, Japan*

Abstract. *SHOX* haploinsufficiency due to mutations in the coding exons or microdeletions involving the coding exons and/or the enhancer regions accounts for approximately 80% and 2–16% of genetic causes of Leri-Weill dyschondrosteosis and idiopathic short stature, respectively. The most characteristic feature in patients with *SHOX* deficiency is Madelung deformity, a cluster of anatomical changes in the wrist that can be attributed to premature epiphyseal fusion of the distal radius. Computed tomography of SHOX-deficient patients revealed a thin bone cortex and an enlarged total bone area at the diaphysis of the radius, while histopathological analyses showed a disrupted columnar arrangement of chondrocytes and an expanded hypertrophic layer of the growth plate. Recent studies have suggested that perturbed programmed cell death of hypertrophic chondrocytes may underlie the skeletal changes related to *SHOX* deficiency. Furthermore, the formation of an aberrant ligament tethering the lunate and radius has been implicated in the development of Madelung deformity. Blood estrogen levels and mutation types have been proposed as phenotypic determinants of *SHOX* deficiency, although other unknown factors may also affect clinical severity of this entity.

Key words: chondrocyte, Leri-Weill dyschondrosteosis, Madelung deformity, short stature, Vickers ligament

Introduction

SHOX (NM_000451.3) encodes a transcription factor exclusively expressed in the developing limb and pharyngeal arch in the human embryo (1). Heterozygous mutations of *SHOX* lead to Leri-Weill dyschondrosteosis characterized by wrist deformity and mesomelic short stature (LWD; OMIM #249700), as well as idiopathic short stature without apparent skeletal malformations (ISS; OMIM # 300582) (2–4). Less specific skeletal changes such as high arched palate, short metacarpals, scoliosis, and micrognathia have also been described in patients with *SHOX* deficiency (5). Previous studies have shown that *SHOX* deficiency accounts for approximately 80% and 2–16% of genetic causes of LWD and ISS, respectively (5–8). Furthermore, haploinsufficiency of *SHOX* represents the major cause of growth failure in patients with Turner syndrome (9). Thus, *SHOX* deficiency is a clinically important condition,

Received: April 8, 2014
Accepted: April 18, 2014
Corresponding author: Dr. Maki Fukami, Department of Molecular Endocrinology, National Research Institute for Child Health and Development, 157-8535 Tokyo, Japan.
E-mail: fukami-m@ncchd.go.jp.

particularly in the field of pediatric endocrinology and orthopedics. This review article introduces our current understanding of the causative mechanisms and phenotypic characteristics of SHOX-associated skeletal malformations.

Molecular Basis of *SHOX* Deficiency

SHOX resides in the short arm pseudoautosomal region of the X and Y chromosomes (PAR1) and escapes X inactivation (2). Thus, although *SHOX* is located on the sex chromosomes, molecular defects of *SHOX* are inherited in an autosomal dominant manner. To date, several point mutations in the *SHOX*-coding exons and submicroscopic deletions encompassing the coding region and/or the upstream or downstream enhancer regions have been identified in more than 200 patients with LWD or ISS (5–9). Previously reported *SHOX* mutations are listed in the SHOX Mutation Database (http://hyg-serv-01.hyg.uni-heidelberg.de/lovd/index.php?select_db=SHOX) (10). The precise position of the *SHOX* enhancers remains to be determined, although the upstream and downstream enhancers have been mapped to a ~300 kb region ~95 kb upstream and an ~30 kb region ~250 kb downstream from the start codon, respectively (11–13). Deletions involving the coding and/or downstream enhancer regions account for about 60% of Japanese LWD patients (14), and those affecting the downstream enhancer regions are the major genetic causes in Spanish patients (15). High recombination frequency and the abundant presence of repeat sequences in PAR1 likely play a role in the development of submicroscopic genomic rearrangements involving *SHOX* (14).

Skeletal Deformity in Patients with *SHOX* Deficiency

The most characteristic feature in patients with *SHOX* deficiency is Madelung deformity, a cluster of anatomical changes in the forearm including bowing and shortening of the radius, prominence of the ulnar head and palmar and ulnar deviation ("pyramidal configuration") of the carpal bones (Fig. 1 and Fig. 2) (16). Clinical manifestations induced by Madelung deformity include wrist pain, deformation and limited joint motion (16, 17). Radiological findings of Madelung deformity include the absence or narrowing of the ulnar portion of the distal radial physis, anterior bowing of the radial shaft and dorsal subluxation of the ulnar head (17, 18). Several additional skeletal changes such as triangularization of the distal radial epiphysis have been associated with

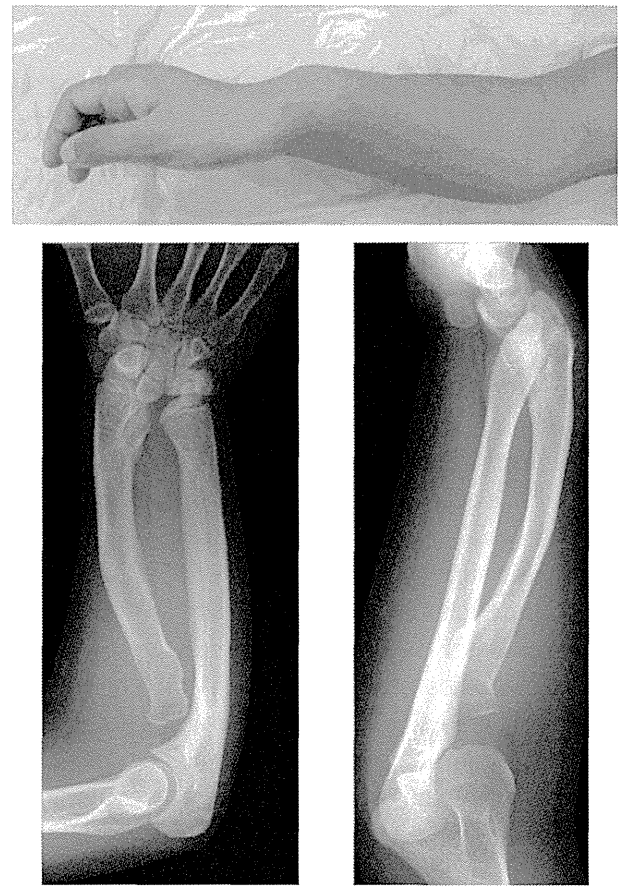

Fig. 1. Madelung deformity in a female patient with *SHOX* deficiency. Upper panel: appearance of the forearm. Prominence of the distal ulna is shown. Lower panel: radiographic findings. Shortening and bowing of the radius and dorsal subluxation of the ulnar head are shown.

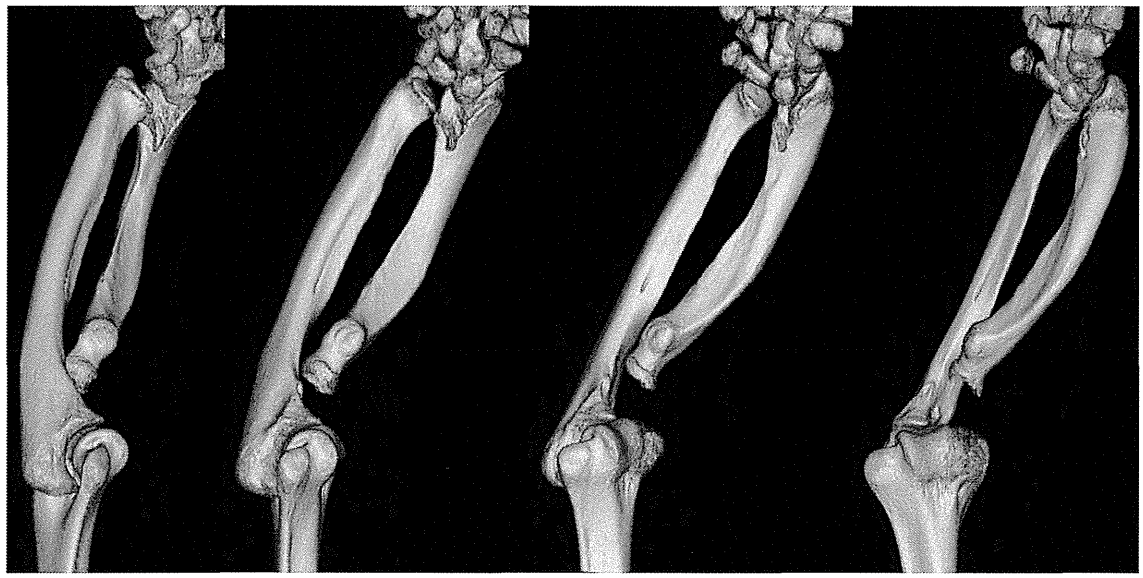

Fig. 2. Forearm three-dimensional computed tomography of a female patient with *SHOX* deficiency. Significant findings include shortening of the radius, pyramidal configuration of the carpal bones and dorsal subluxation of the ulna in addition to severely disturbed structural organization of the elbow joint.

Madelung deformity (19). In patients with severe Madelung deformity, the structural organization of the elbow joint is also disrupted (Fig. 2).

Previous studies have suggested that the primary lesion of Madelung deformity is the premature epiphyseal fusion at the volar-ulnar portion of the radial growth plate (20). Impaired growth of the radius due to the early epiphyseal fusion, in combination with relatively preserved growth of the ulna, appears to underlie the characteristic deformity (18, 21). The appearance of the wrist varies among patients and probably depends on the fusion position along the anterior-posterior axis of the radial epiphysis (22).

Although Madelung deformity usually occurs as a result of *SHOX* deficiency, it can also take place as a component of other congenital disorders such as multiple exostoses syndrome, multiple epiphyseal dysplasia and dysostosis multiplex of mucopolysaccharidosis (23). Madelung-like deformity has also been observed in patients with pseudohypoparathyroidism type 1b (24). In addition, Madelung deformity can occur as a change secondary to injury and infection (19).

When Madelung deformity is accompanied by short stature and mesomelic shorting of the limbs, it is referred to as LWD. The mesomelic short stature of LWD can be explained as a result of impaired linear growth of the radius, ulna, tibia and fibula. A decreased extremity/trunk ratio with a fairly preserved sitting height and head circumference is a characteristic auxological finding of patients with LWD (5, 25). Rappold et al. developed a phenotype scoring system for screening of individuals with possible *SHOX* deficiency from patients with short stature (8). They suggested the following eight clinical features as indicators for *SHOX* deficiency: arm span/height ratio, sitting height/height ratio, body mass index, cubitus valgus, short forearm, bowing of the forearm, muscular hypertrophy and dislocation of the ulna. Although *SHOX* deficiency is the only condition that has been implicated in LWD, *SHOX* abnormalities have been detected only in 50–90% of patients with LWD (5). It remains currently unknown whether LWD patients with apparently normal *SHOX* alleles have mutations in the regulatory regions

of *SHOX* or in a hitherto unidentified gene involved in skeletal development.

Changes in Bone Geometry and Bone Mineral Density

Using peripheral quantitative computed tomography of the forearm, Soucek *et al.* investigated bone mineral density and bone geometry in 10 prepubertal patients with *SHOX* deficiency and 22 patients with Turner syndrome (26). They found that patients of both groups had a thin bone cortex and an enlarged total bone area at the diaphysis of the radius compared with control individuals. On the other hand, these patients had a normal trabecular bone mineral density and bone strength index. Soucek *et al.* proposed that the skeletal changes observed in patients with *SHOX* deficiency are attributable to an adjustment of the long bones with a disrupted cortex to the mechanical loading that aims to increase bone strength.

Histopathological Changes

Munns *et al.* investigated histopathological findings of the surgically-excised growth plate of the distal radius obtained from two patients with molecularly confirmed *SHOX* deficiency (22). They found disrupted columnar arrangement of chondrocytes; the normal tandem stacking of mature chondrocytes within columns was replaced by a side-by-side arrangement. Furthermore, the presence of hypertrophic osteoid with micro-enchondromata in the metaphysis suggested aberrant endochondral ossification. Significant expansion of the hypertrophic layer and reduction of the proliferative layer were observed in the growth plate. These data imply that the SHOX protein is required for ordered zonal development of chondrocytes. In this regard, *SHOX* is strongly expressed in terminally differentiated hypertrophic chondrocytes and less obviously in proliferating and reserve chondrocytes (27). *In vitro* assays with osteosarcoma cells indicated that SHOX induces oxidative stress and activates the intrinsic apoptotic pathway (27). Thus, it is possible that SHOX plays a critical role in chondrocyte development by regulating the cell cycle and apoptosis of hypertrophic chondrocytes. Indeed, premature epiphyseal fusion in patients with *SHOX* deficiency may reflect a perturbed cell death process in the growth plate. To date, however, the precise mechanism by which SHOX exerts its effect on chondrocyte development remains unknown. Although *in vivo* and *in vitro* assays have indicated that several proteins such as BNP, FGFR3, SOX5, and SOX6 can interact with SHOX (28, 29), the function of SHOX in human tissues has yet to be elucidated.

Formation of an Abnormal Ligament

Vickers and Nielsen identified an abnormal ligament in patients with Madelung deformity (30). The "Vickers ligament" tethers the lunate to the distal portion of the radius and can have a diameter as large as 8 mm (Fig. 3) (22). Histological analysis demonstrated that the Vickers ligament is a morphologically normal ligament consisting of collagen and elastin fibers (22). This ligament is predicted to promote pyramidal configuration of the carpal bones by disturbing the physiological migration of these bones during growth (22). Furthermore, this ligament may exert an inhibitory effect on linear growth of the radius by compressing its distal epiphysis (18). Therefore, it is possible that the Vickers ligament constitutes an essential factor in the development of Madelung deformity. Although the process by which the Vickers ligament forms has yet to be clarified, this ligament is regarded as a secondary change of the forearm deformity (30, 31). Actually, the ligament seems to consist of hypertrophied connective tissues that form under a mechanical force that arises from asymmetrical growth of the radius and ulna.

Recent advancements in high-resolution magnetic resonance imaging have enabled early

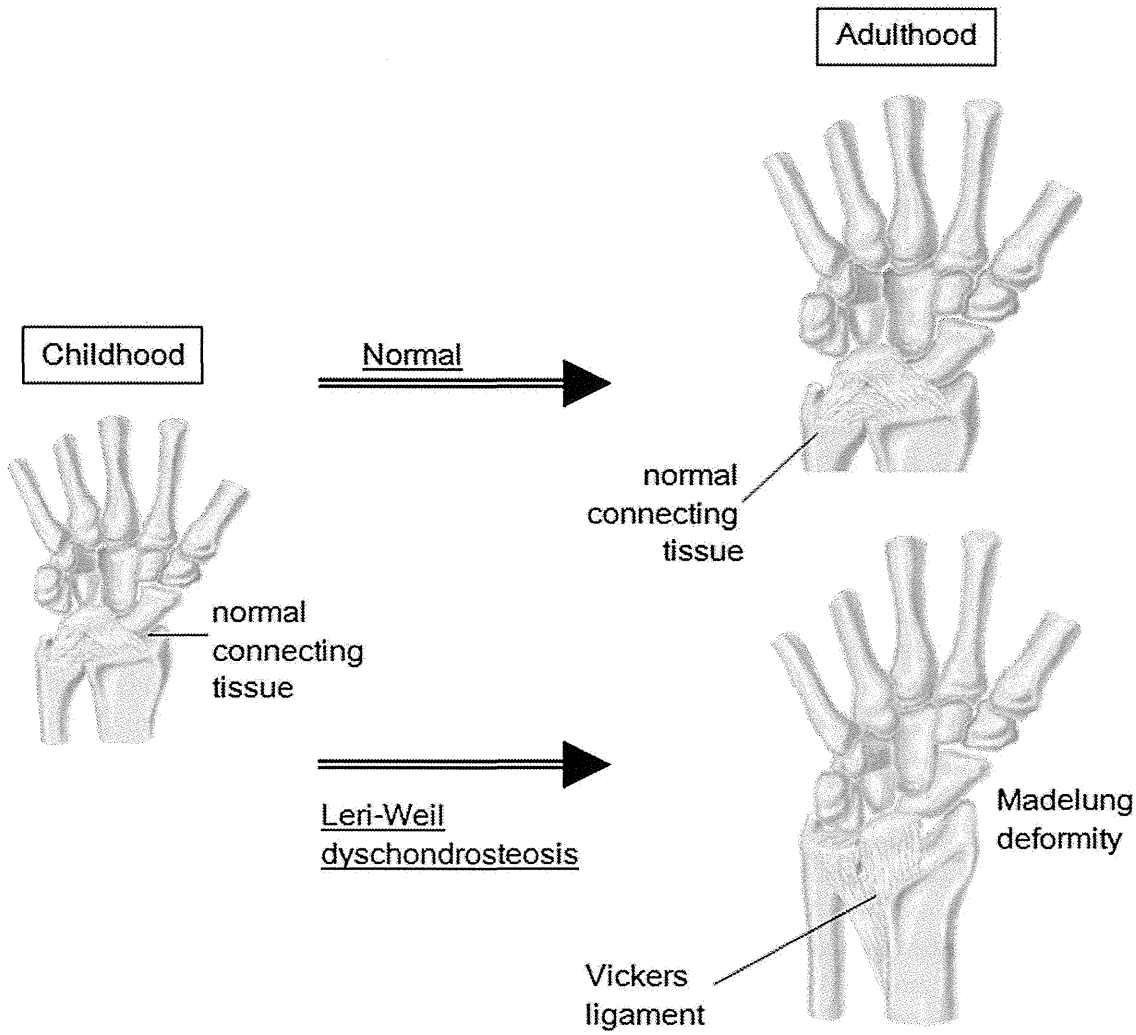

Fig. 3. Schematic representation of the Vickers ligament that tethers the lunate to the distal portion of the radius. This ligament seems to consist of hypertrophied connective tissues that form under the mechanical force that arises from asymmetrical growth of the radius and ulna.

detection of the Vickers ligament (31). Previous studies have indicated that surgical removal of the Vickers ligament in combination with dome osteotomy is beneficial to patients with Madelung deformity; Harley *et al.* reported that these surgical interventions effectively improved the clinical features of adolescent patients with Madelung deformity (21), while Steinman *et al.* showed that these interventions can provide long-term correction of the wrist deformity (16). However, since the number of treated patients was small, further studies are necessary to validate these findings.

Phenotypic Determinants

Skeletal changes of *SHOX* deficiency tend to be more severe in adult females than in children or adult males (5). Obvious Madelung deformity is rare in prepubertal patients, although decreased extremity/trunk ratios and subtle skeletal changes are observed in the majority

of children with *SHOX* deficiency (5, 32, 33). These data can be explained by assuming that estrogens exert a deleterious effect on skeletal formation in patients with *SHOX* abnormalities. Since estrogens induce physiological skeletal maturation in both sexes (34), they may also enhance premature epiphyseal fusion in patients with *SHOX* deficiency. Consistent with this, severe Madelung deformity is rarely seen in Turner females in whom ovarian function is frequently impaired (35). Furthermore, a longitudinal study of a female patient with *SHOX* deficiency and normal ovarian function showed age-appropriate skeletal maturation before puberty and rapidly advanced bone age during puberty (36). On the other hand, since Soucek et al. revealed a significant difference in bone geometry between prepubertal patients with the 46,XX karyotype and prepubertal Turner females, it is likely that some factors other than estrogens may also underlie relatively mild skeletal features in Turner females (26). Soucek et al. suggested karyotype mosaicism as one of the possible candidates for such factors (26). It is known that the karyotype of Turner females is heterogeneous and includes 45,X/46,XX. Since two normal *SHOX* alleles are present in a certain percentage of cells in females with the 45,X/46,XX karyotype, this may lead to relatively well preserved skeletal structures in such patients.

Mutation types may affect the phenotypic severity of *SHOX* deficiency. It has been proposed that molecular defects involving only the enhancer regions are associated with broader phenotypic variation than deletions/mutations affecting the coding exons; Chen et al. have described more severe skeletal changes in patients with enhancer deletions than in those with mutations/deletions affecting the coding exons (37), while Rosilio et al. reported relatively mild phenotypes in patients with enhancer deletions (7). On the other hand, no apparent genotype-phenotype correlation has been reported for *SHOX* intragenic mutations/deletions (8).

Conclusion

Recent studies have indicated that *SHOX* deficiency leads to premature epiphyseal fusion at the distal radius, possibly by disturbing programmed cell death of hypertrophic chondrocytes. In addition, the formation of an aberrant ligament tethering the lunate and radius appears to play a role in the development of Madelung deformity. Blood estrogen levels and mutation types have been proposed as phenotypic determinants of *SHOX* deficiency, although other unknown factors may also modify the clinical severity of this condition.

Acknowledgements

This work was supported by a Grant-in-Aid and for Scientific Research on Innovative Areas from the Ministry of Education, Culture, Sports, Science and Technology, by a Grant for Research on Intractable Diseases from the Ministry of Health, Labor and Welfare and by Grants from the National Center for Child Health and Development and from the Takeda foundation.

References

1. Clement-Jones M, Schiller S, Rao E, Blaschke RJ, Zuniga A, Zeller R, *et al.* The short stature homeobox gene SHOX is involved in skeletal abnormalities in Turner syndrome. Hum Mol Genet 2000;9: 695–702. [Medline] [CrossRef]
2. Rao E, Weiss B, Fukami M, Rump A, Niesler B, Mertz A, *et al.* Pseudoautosomal deletions encompassing a novel homeobox gene cause growth failure in idiopathic short stature and Turner syndrome. Nat Genet 1997;16: 54–63. [Medline] [CrossRef]
3. Shears DJ, Vassal HJ, Goodman FR, Palmer RW, Reardon W, Superti-Furga A, *et al.* Mutation and deletion of the pseudoautosomal gene SHOX cause Leri-Weill dyschondrosteosis. Nat Genet 1998;19: 70–3. [Medline] [CrossRef]
4. Belin V, Cusin V, Viot G, Girlich D, Toutain A, Moncla A, *et al.* SHOX mutations in

dyschondrosteosis (Leri-Weill syndrome). Nat Genet 1998;19: 67–9. [Medline] [CrossRef]
5. Binder G. Short stature due to SHOX deficiency: genotype, phenotype, and therapy. Horm Res Paediatr 2011;75: 81–9. [Medline] [CrossRef]
6. Rappold GA, Fukami M, Niesler B, Schiller S, Zumkeller W, Bettendorf M, *et al.* Deletions of the homeobox gene SHOX (short stature homeobox) are an important cause of growth failure in children with short stature. J Clin Endocrinol Metab 2002;87: 1402–6. [Medline] [CrossRef]
7. Rosilio M, Huber-Lequesne C, Sapin H, Carel JC, Blum WF, Cormier-Daire V. Genotypes and phenotypes of children with SHOX deficiency in France. J Clin Endocrinol Metab 2012;97: E1257–65. [Medline] [CrossRef]
8. Rappold G, Blum WF, Shavrikova EP, Crowe BJ, Roeth R, Quigley CA, *et al.* Genotypes and phenotypes in children with short stature: clinical indicators of SHOX haploinsufficiency. J Med Genet 2007;44: 306–13. [Medline] [CrossRef]
9. Ross JL, Scott C Jr, Marttila P, Kowal K, Nass A, Papenhausen P, *et al.* Phenotypes Associated with SHOX Deficiency. J Clin Endocrinol Metab 2001;86: 5674–80. [Medline] [CrossRef]
10. Niesler B, Röth R, Wilke S, Fujimura F, Fischer C, Rappold G. The novel human SHOX allelic variant database. Hum Mutat 2007;28: 933–8. [Medline] [CrossRef]
11. Benito-Sanz S, Thomas NS, Huber C, Gorbenko del Blanco D, Aza-Carmona M, Crolla JA, *et al.* A novel class of Pseudoautosomal region 1 deletions downstream of SHOX is associated with Leri-Weill dyschondrosteosis. Am J Hum Genet 2005;77: 533–44. [Medline] [CrossRef]
12. Benito-Sanz S, Aza-Carmona M, Rodríguez-Estevez A, Rica-Etxebarria I, Gracia R, Campos-Barros A, *et al.* Identification of the first PAR1 deletion encompassing upstream SHOX enhancers in a family with idiopathic short stature. Eur J Hum Genet 2012;20: 125–7. [Medline] [CrossRef]
13. Fukami M, Kato F, Tajima T, Yokoya S, Ogata T. Transactivation function of an approximately 800-bp evolutionarily conserved sequence at the SHOX 3′ region: implication for the downstream enhancer. Am J Hum Genet 2006;78: 167–70. [Medline] [CrossRef]
14. Fukami M, Dateki S, Kato F, Hasegawa Y, Mochizuki H, Horikawa R, *et al.* Identification and characterization of cryptic SHOX intragenic deletions in three Japanese patients with Léri-Weill dyschondrosteosis. J Hum Genet 2008;53: 454–9. [Medline] [CrossRef]
15. Benito-Sanz S, del Blanco DG, Aza-Carmona M, Magano LF, Lapunzina P, Argente J, *et al.* PAR1 deletions downstream of SHOX are the most frequent defect in a Spanish cohort of Léri-Weill dyschondrosteosis (LWD) probands. Hum Mutat 2006;27: 1062. [Medline] [CrossRef]
16. Steinman S, Oishi S, Mills J, Bush P, Wheeler L, Ezaki M. Volar ligament release and distal radial dome osteotomy for the correction of Madelung deformity: long-term follow-up. J Bone Joint Surg Am 2013;95: 1198–204. [Medline] [CrossRef]
17. Schmidt-Rohlfing B, Schwöbel B, Pauschert R, Niethard FU. Madelung deformity: clinical features, therapy and results. J Pediatr Orthop B 2001;10: 344–8. [Medline]
18. Ghatan AC, Hanel DP. Madelung deformity. J Am Acad Orthop Surg 2013;21: 372–82. [Medline] [CrossRef]
19. Felman AH, Kirkpatrick Jr JA. Madelung's deformity: observations in 17 patients. Radiology 1969;93: 1037–42. [Medline]
20. Henry A, Thorburn MJ. Madelung's deformity. A clinical and cytogenetic study. J Bone Joint Surg Br 1967;49: 66–73. [Medline]
21. Harley BJ, Brown C, Cummings K, Carter PR, Ezaki M. Volar ligament release and distal radius dome osteotomy for correction of Madelung's deformity. J Hand Surg Am 2006;31: 1499–506. [Medline] [CrossRef]
22. Munns CF, Glass IA, LaBrom R, Hayes M, Flanagan S, Berry M, *et al.* Histopathological analysis of Leri-Weill dyschondrosteosis: disordered growth plate. Hand Surg 2001;6: 13–23. [Medline] [CrossRef]
23. Ty JM, James MA. Failure of differentiation: Part II (arthrogryposis, camptodactyly, clinodactyly, madelung deformity, trigger finger, and trigger thumb). Hand Clin 2009;25: 195–213. [Medline] [CrossRef]
24. Sanchez J, Perera E, Jan de Beur S, Ding C, Dang

A, Berkovitz GD, *et al.* Madelung-like deformity in pseudohypoparathyroidism type 1b. J Clin Endocrinol Metab 2011;96: E1507–11. [Medline] [CrossRef]
25. Wolters B, Lass N, Wunsch R, Böckmann B, Austrup F, Reinehr T. Short stature before puberty: which children should be screened for SHOX deficiency? Horm Res Paediatr 2013;80: 273–80. [Medline] [CrossRef]
26. Soucek O, Zapletalova J, Zemkova D, Snajderova M, Novotna D, Hirschfeldova K, *et al.* Prepubertal girls with Turner syndrome and children with isolated SHOX deficiency have similar bone geometry at the radius. J Clin Endocrinol Metab 2013;98: E1241–7. [Medline] [CrossRef]
27. Hristov G, Marttila T, Durand C, Niesler B, Rappold GA, Marchini A. SHOX triggers the lysosomal pathway of apoptosis via oxidative stress. Hum Mol Genet 2014;23: 1619–30. [Medline] [CrossRef]
28. Rappold GA, Durand C, Decker E, Marchini A, Schneider KU. New roles of SHOX as regulator of target genes. Pediatr Endocrinol Rev 2012;9(Suppl 2): 733–8. [Medline]
29. Aza-Carmona M, Shears DJ, Yuste-Checa P, Barca-Tierno V, Hisado-Oliva A, Belinchón A, *et al.* SHOX interacts with the chondrogenic transcription factors SOX5 and SOX6 to activate the aggrecan enhancer. Hum Mol Genet 2011;20: 1547–59. [Medline] [CrossRef]
30. Vickers D, Nielsen G. Madelung deformity: surgical prophylaxis (physiolysis) during the late growth period by resection of the dyschondrosteosis lesion. J Hand Surg [Br] 1992;17: 401–7. [Medline] [CrossRef]
31. Stehling C, Langer M, Nassenstein I, Bachmann R, Heindel W, Vieth V. High resolution 3.0 Tesla MR imaging findings in patients with bilateral Madelung's deformity. Surg Radiol Anat 2009;31: 551–7. [Medline] [CrossRef]
32. Kosho T, Muroya K, Nagai T, Fujimoto M, Yokoya S, Sakamoto H, *et al.* Skeletal features and growth patterns in 14 patients with haploinsufficiency of SHOX: implications for the development of Turner syndrome. J Clin Endocrinol Metab 1999;84: 4613–21. [Medline] [CrossRef]
33. Ogata T, Matsuo N, Nishimura G. SHOX haploinsufficiency and overdosage: impact of gonadal function status. J Med Genet 2001;38: 1–6. [Medline] [CrossRef]
34. Emons J, Chagin AS, Sävendahl L, Karperien M, Wit JM. Mechanisms of growth plate maturation and epiphyseal fusion. Horm Res Paediatr 2011;75: 383–91. [Medline] [CrossRef]
35. Binder G, Fritsch H, Schweizer R, Ranke MB. Radiological signs of Leri-Weill dyschondrosteosis in Turner syndrome. Horm Res 2001;55: 71–6. [Medline] [CrossRef]
36. Fukami M, Matsuo N, Hasegawa T, Sato S, Ogata T. Longitudinal auxological study in a female with SHOX (short stature homeobox containing gene) haploinsufficiency and normal ovarian function. Eur J Endocrinol 2003;149: 337–41. [Medline] [CrossRef]
37. Chen J, Wildhardt G, Zhong Z, Röth R, Weiss B, Steinberger D, *et al.* Enhancer deletions of the SHOX gene as a frequent cause of short stature: the essential role of a 250 kb downstream regulatory domain. J Med Genet 2009;46: 834–9. [Medline] [CrossRef]

Journal of Human Genetics (2014) 59, 353–356

www.nature.com/jhg

SHORT COMMUNICATION

Mutation spectrum and phenotypic variation in nine patients with *SOX2* abnormalities

Junichi Suzuki[1,2], Noriyuki Azuma[3], Sumito Dateki[1,4], Shun Soneda[1], Koji Muroya[5], Yukiyo Yamamoto[6], Reiko Saito[6], Shinichiro Sano[1], Toshiro Nagai[7], Hiroshi Wada[8], Akira Endo[9], Tatsuhiko Urakami[2], Tsutomu Ogata[1,10] and Maki Fukami[1]

Multiple mutations in *SOX2* have been identified in patients with ocular anomalies and/or pituitary dysfunction. Here, we identified *SOX2* abnormalities in nine patients. The molecular defects included one missense, one nonsense and four frameshift mutations, and three submicroscopic deletions involving *SOX2*. Three of the six mutations and all deletions were hitherto unreported. The breakpoints determined in one deletion were located within *Alu* repeats and accompanied by an overlap of 11 bp. Three of the six mutations encoded SOX2 proteins that lacked *in vitro* transactivation activity for the *HESX1* promoter, whereas the remaining three generated proteins with ~15–~20% of transactivation activity. All cases manifested ocular anomalies of various severities, together with several complications including arachnoid cyst and hamartoma. There was no apparent correlation between the residual activity and clinical severity. The results indicate that molecular defects in *SOX2* are highly variable and include *Alu* repeat-mediated genomic rearrangements. Our data provide further evidence for wide phenotypic variation of *SOX2* abnormalities and the lack of genotype–phenotype correlation in patients carrying *SOX2* lesions.
Journal of Human Genetics (2014) **59,** 353–356; doi:10.1038/jhg.2014.34; published online 8 May 2014

Keywords: anophthalmia; deletion; genotype-phenotype correlation; microphthalmia; mutation; SOX2

SOX2 (NP_003097.1) has a critical role in the development of the eye, pituitary and central nervous system through transactivation of multiple genes including *HESX1*.[1–3] Haploinsufficiency of *SOX2* (NM_003106.3) leads to anophthalmia/microphthalmia and pituitary dysfunction, in addition to various neuronal defects such as mental retardation, brain malformation and hearing loss.[3–7] Although >80 patients with *SOX2* abnormalities have been reported to date,[3–11] current understanding of mutation spectrum and phenotypic determinants of this condition remains fragmentary. In fact, *in vitro* functional assays have been performed only for a small number of mutations.[3,11–13] Furthermore, whereas Kelberman *et al.*[12] found no obvious genotype–phenotype correlation, Schneider *et al.*[14] reported that patients with missense mutations had milder ocular phenotypes than those with nonsense or frameshift mutations.

Here, we identified *SOX2* abnormalities in nine patients (cases 1–9). This study was approved by the Institutional Review Board Committee at the National Center for Child Health and Development and performed after obtaining written informed consent. Molecular defects in cases 1–9 were identified through sequencing and copy number analyses of *SOX2* for 37 patients with ocular anomalies and 15 patients with pituitary dysfunction and normal eyes (Supplementary Table S1). Mutations in the coding region were examined by direct sequencing, and copy number abnormalities were analyzed by multiplex ligation-dependent amplification and array comparative genomic hybridization. Detailed methods are provided in Supplementary Table S2. Cases 1–6 carried heterozygous intragenic mutations, whereas cases 7–9 had heterozygous submicroscopic deletions involving *SOX2*. Cases 1–9 invariably manifested developmental defects of eyes, in addition to multiple complications including arachnoid cyst and hamartoma (Table 1 and Supplementary Table S3). The ocular phenotypes included unilateral and bilateral microphthalmia, bilateral coloboma and bilateral anophthalmia. The *SOX2* mutations in cases 1–6 consisted of three previously reported mutations (c.70_89del20, c.70_86del17 and c.480C>G) and three novel mutations (c.235T>C, c.244_245delTT and c.402delC) (Figure 1a). None of the six mutations have been registered as polymorphisms in the single-nucleotide polymorphism database (dbSNP, http://www.ncbi.nlm.nih.gov/). *In vitro* reporter assays using a vector containing the *HESX1* promoter indicated that c.235T>C, c.402delC and c.480C>G encoded proteins with ~15–

[1]National Research Institute for Child Health and Development, Department of Molecular Endocrinology, Tokyo, Japan; [2]Department of Pediatrics and Child Health, Nihon University School of Medicine, Tokyo, Japan; [3]National Center for Child Health and Development, Department of Ophthalmology and Laboratory of Cell Biology, Tokyo, Japan; [4]Department of Pediatrics, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan; [5]Kanagawa Children's Medical Center, Department of Endocrinology and Metabolism, Yokohama, Japan; [6]Department of Pediatrics, School of Medicine, University of Occupational and Environmental Health, Fukuoka, Japan; [7]Department of Pediatrics, Koshigaya Hospital, Dokkyo Medical University, Koshigaya, Japan; [8]Department of Pediatrics, Yodogawa Christian Hospital, Osaka, Japan; [9]Department of Pediatrics, Iwata City Hospital, Iwata, Japan and [10]Department of Pediatrics, Hamamatsu University School of Medicine, Hamamatsu, Japan
Correspondence: Dr M Fukami, National Research Institute for Child Health and Development, Department of Molecular Endocrinology, Tokyo 157-8535, Japan.
E-mail: fukami-m@ncchd.go.jp
Received 23 January 2014; revised 9 April 2014; accepted 10 April 2014; published online 8 May 2014

Table 1 Molecular and clinical findings of cases 1–9

	Case 1	*Case 2*	*Case 3*	*Case 4*	*Case 5*	*Case 6*	*Case 7*	*Case 8*	*Case 9*
Age at examination	3 Months	13 Days	1 Year	19 Years	7 Years	1 Year	12 Years	1 Year	19 Years
Sex	Male	Male	Female	Female	Female	Female	Female	Male	Male
Molecular findings									
SOX2 mutation (cDNA)	c.235T>C	c.480C>G	c.70_89del20[a]	c.70_86del17[b]	c.244_245delTT	c.402delC	Gene deletion	Gene deletion	Gene deletion
SOX2 mutation (protein)	p.W79R	p.Y160X	p.N24fsX88	p.N24fsX89	p.L82fsX94	p.G135fsX153	Deletion	Deletion	Deletion
Parental origin of the mutation/deletion	NE	NE	*De novo*	NE	*De novo*	Father mosaic	NE	NE	*De novo*
In vitro transactivating activity	Hypomorphic	Hypomorphic	Amorphic	Amorphic	Amorphic	Hypomorphic	Null	Null	Null
Clinical findings									
Ocular abnormality (right)	MO	AO	AO	AO	severe MO	MO	severe MO	AO	Retinal fold
Ocular abnormality (left)	Normal	AO	AO	AO	severe MO	MO	severe MO	AO	Optic disc coloboma
Developmental delay	No	Unknown	No	No	Yes	Yes	Yes	Yes	Yes
Prenatal growth	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	LGA
Postnatal growth failure	Yes	Unknown	Yes	Yes	Yes	Yes	Yes	Yes	Normal
Other complication	Esophageal atresia	None	None	None	Hemi-hypertrophy	None	Seizure	None	None
Pituitary hormone deficiency	LH, FSH	NE	NE	LH, FSH, GH	NE	NE	LH, FSH,GH	LH, FSH, GH	LH, FSH
Brain MRI findings	Arachnoid cyst	Dandy–Walker syndrome	Hamartoma	Pituitary hypoplasia	Normal	White matter signal abnormality	Ectopic posterior lobe	Pituitary hypoplasia	NE

Abbreviations: AO, anophthalmia; FSH, follicle-stimulating hormone; GH, growth hormone; LGA, large for gestational age; LH, luteinizing hormone; MO, microphthalmia; MRI, magnetic resonance imaging; NE, not examined.
[a]delAACTCCACCGCGGCGGCGGC.
[b]delAACTCCACCGCGGCGGC.

~20% transactivation activity, whereas the remaining three generated proteins that lacked the activity (Figure 1b). Of the six mutations, c.70_89del20, c.70_86del17 and c.244_245delTT affected the possible nuclear localization signals predicted from mouse data, and therefore seemed to disturb the intracellular localization of the SOX2 protein (Figure 1a).[15] Furthermore, it remained possible that mRNAs of the nonsense and frameshift mutations undergo early degradation *in vivo*. The deletions in cases 7–9 affected ~1.0 to ~2.5 Mb genomic regions at 3q26-27 including *SOX2*. These deletions overlapped with, but were not identical to, previously reported deletions (Figure 2).[4,7–9,12–14,16] Sequences at the fusion junction were characterized in case 7, showing that the two breakpoints resided within *Alu* repeats and shared an overlap of 11 bp (Figure 2).

Several matters are noteworthy. First, molecular lesions in cases 1–9 were heterogeneous and included six point mutations and three submicroscopic deletions. These data support a broad mutation spectrum of *SOX2* abnormalities.[8,9] Notably, we identified submicroscopic deletions involving *SOX2* in three cases. As multiple microdeletions at 3q26-27 have been reported in patients with ocular anomalies (Figure 2),[4,7–9,12–14,16] it is possible that the genomic region around *SOX2* represents a hotspot for chromosomal rearrangements. In this regard, it is noteworthy that the sequence at the fusion junction in case 7 is consistent with non-allelic homologous recombination that occurs between two homologous sequences or replication-based errors that are usually associated with microhomology at the fusion junction.[17] Furthermore, the two breakpoints of this deletion resided within *Alu* repeats, which facilitate both recombination- and replication-mediated errors.[18,19] These data imply that *Alu* repeats may have a role in the high frequency of deletions at 3q26-27. Second, cases 1–9 manifested several complications in addition to ocular abnormalities. Importantly, seven cases manifested brain anomalies including arachnoid cyst and hamartoma. In this regard, Alatzoglou *et al.*[13] recently described pituitary tumors in two patients with *SOX2* haploinsufficiency. Thus, *SOX2* abnormalities seem to be associated with various types of developmental defects and tumors in the brain. Third, *SOX2* lesions were identified in 9 of 37 patients with ocular abnormalities, but were absent from 15 patients with pituitary dysfunction and normal eyes. The results are consistent with the previous reports that *SOX2* abnormalities account for 10–20% of the etiology of anophthalmia/microphthalmia and rarely result in pituitary dysfunction without eye abnormalities.[4,5,8,9,11] These data can be explained by assuming that, during development, the eye is highly sensitive to reduced activity of SOX2. Nevertheless, as gonadotropin deficiency was observed in all of our mutation-positive cases, SOX2 appears to be indispensable for the function of the hypothalamus–pituitary axis. Lastly, no apparent genotype–phenotype correlation was found in our patient cohort. Although ocular phenotypes were relatively mild in cases 1 and 6 with hypomorphic mutations and obviously severe in cases 3 and 4 with amorphic mutations, bilateral anophthalmia was also observed in case 2 with a hypomorphic mutation and mild coloboma with normal visual activity was seen in case 9 with *SOX2* deletion. Similarly, the occurrence of pituitary dysfunction, mental retardation and short stature was not associated with the mutation types. The lack of correlation between residual activity and phenotypic severity is consistent with the previously proposed notion that haploinsufficiency of developmental genes is usually associated with a wide range of penetrance and expressivity.[20]

Collectively, the present study argues for a broad spectrum of *SOX2* lesions and indicates for the first time that *Alu* repeat-mediated genomic rearrangements at 3q26-27 account for a part of the etiology

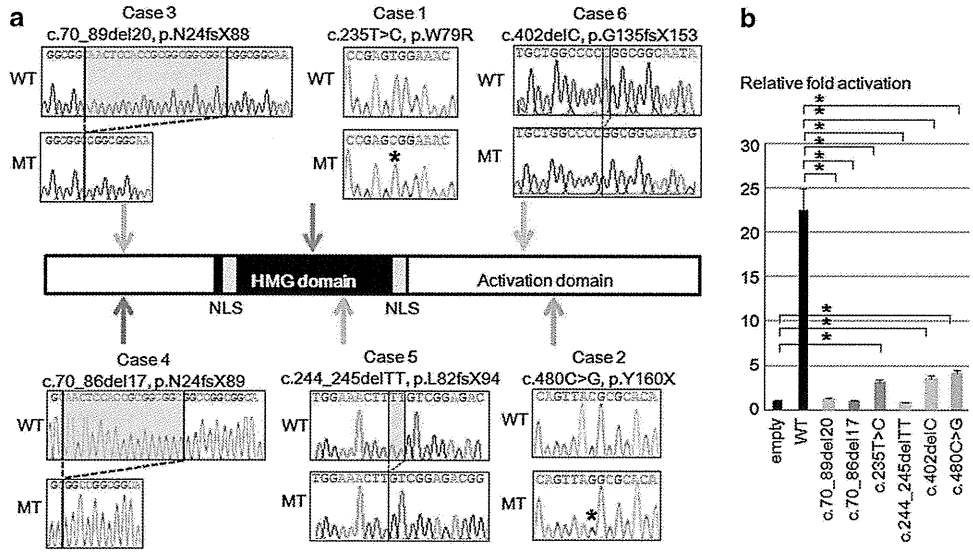

Figure 1 The position and *in vitro* function of *SOX2* mutations identified in the present study. (**a**) Chromatograms of the mutations. Subcloned wild-type (WT) and mutant (MT) sequences are shown. Deleted nucleotides are shaded in gray and mutated nucleotides are indicated by asterisks. The yellow boxes indicate the position of two putative nuclear localization signals (NLSs) predicted from mouse data.[15] (**b**) *In vitro* reporter assay using a luciferase vector containing the *HESX1* promoter. The results are expressed as the mean ± 1 s.d. Asterisks indicate the statistical significance of the results ($P \leqslant 0.05$). The relative fold activation of c.70_89del20, c.70_86del17 and c.244_245delTT was similar to that of the empty vector (empty). The relative percentages of fold activation of c.235T>C, c.402delC and c.480C>G to that of the WT *SOX2* were 13.8%, 15.4% and, 18.4%, respectively.

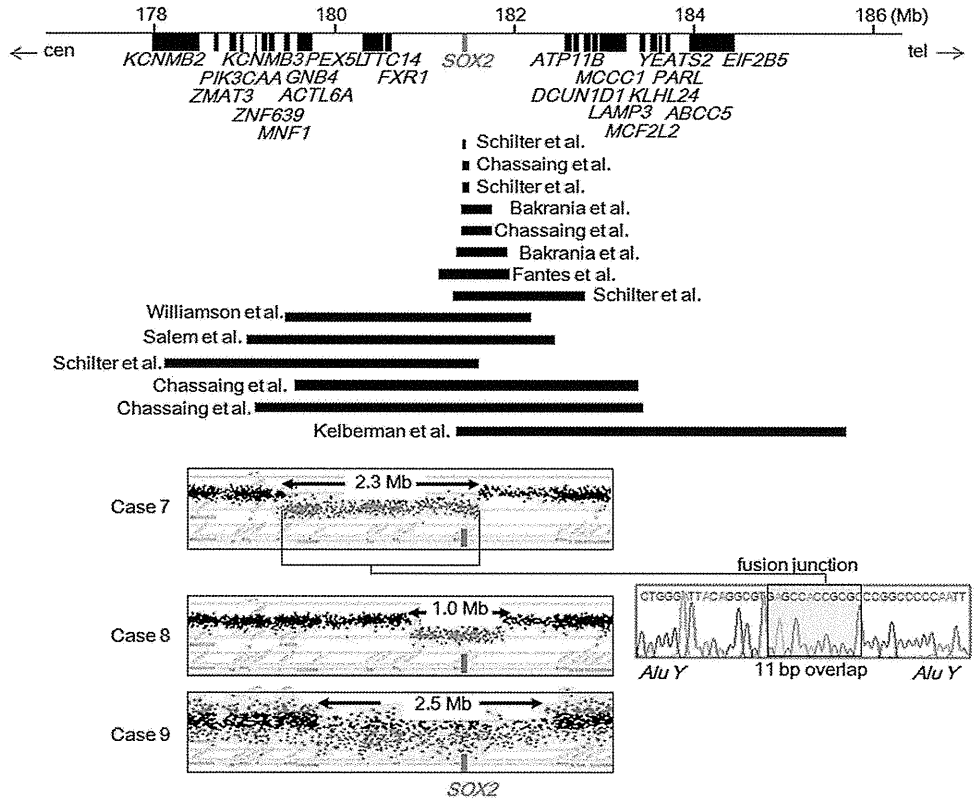

Figure 2 Submicroscopic deletions identified in the present and previous studies. Upper panel: genomic structure of the 3q26-27 region and the position of previously reported deletions. The position of *SOX2* is indicated by a red box and the deletions are depicted by black bars. Genomic positions of the genes and deletions refer to NCBI database (http://www.ncbi.nlm.nih.gov/). Cen, centromere; tel, telomere. Lower panel: comparative genomic hybridization analysis of cases 7–9 from the present study and the sequence at the fusion junction in case 7. The black, red and green dots denote signals indicative of the normal, increased ($> +0.5$) and decreased (< -1.0) copy numbers, respectively. Estimated sizes and positions of the heterozygous deletions are shown.

of *SOX2* haploinsufficiency. Our data provide further evidence for the wide phenotypic variation and the lack of genotype–phenotype correlation in patients with *SOX2* abnormalities.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

This work was supported by grants from the Ministry of Health, Labor and Welfare and from Takeda Science Foundation, by Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science, by Grant-in-Aid for Scientific Research on Innovative Areas from the Ministry of Education, Culture, Sports, Science and Technology and by the Grant of National Center for Child Health and Development.

1 Kelberman, D., Rizzoti, K., Lovell-Badge, R., Robinson, I. C. & Dattani, M. T. Genetic regulation of pituitary gland development in human and mouse. *Endocr. Rev.* **30,** 790–829 (2009).
2 Jayakody, S. A., Andoniadou, C. L., Gaston-Massuet, C., Signore, M., Cariboni, A., Bouloux, P. M. *et al.* SOX2 regulates the hypothalamic-pituitary axis at multiple levels. *J. Clin. Invest.* **122,** 3635–3646 (2012).
3 Kelberman, D., Rizzoti, K., Avilion, A., Bitner-Glindzicz, M., Cianfarani, S., Collins, J. *et al.* Mutations within Sox2/SOX2 are associated with abnormalities in the hypothalamo-pituitary-gonadal axis in mice and humans. *J. Clin. Invest.* **116,** 2442–2455 (2006).
4 Fantes, J., Ragge, N. K., Lynch, S. A., McGill, N. I., Collin, J. R., Howard-Peebles, P. N. *et al.* Mutations in SOX2 cause anophthalmia. *Nat. Genet.* **33,** 461–463 (2003).
5 Stark, Z., Storen, R., Bennetts, B., Savarirayan, R. & Jamieson, R. V. Isolated hypogonadotropic hypogonadism with SOX2 mutation and anophthalmia/microphthalmia in offspring. *Eur. J. Hum. Genet.* **19,** 753–756 (2011).
6 Hagstrom, S. A., Pauer, G. J., Reid, J., Simpson, E., Crowe, S., Maumenee, I. H. *et al.* SOX2 mutation causes anophthalmia, hearing loss, and brain anomalies. *Am. J. Med. Genet. A* **138A,** 95–98 (2005).
7 Williamson, K. A., Hever, A. M., Rainger, J., Rogers, R. C., Magee, A., Fiedler, Z. *et al.* Mutations in SOX2 cause anophthalmia-esophageal-genital (AEG) syndrome. *Hum. Mol. Genet.* **15,** 1413–1422 (2006).
8 Bakrania, P., Robinson, D. O., Bunyan, D. J., Salt, A., Martin, A., Crolla, J. A. *et al.* SOX2 anophthalmia syndrome: 12 new cases demonstrating broader phenotype and high frequency of large gene deletions. *Br. J. Ophthalmol.* **91,** 1471–1476 (2007).
9 Chassaing, N., Causse, A., Vigouroux, A., Delahaye, A., Alessandri, J. L., Boespflug-Tanguy, O. *et al.* Molecular findings and clinical data in a cohort of 150 patients with anophthalmia/microphthalmia. *Clin. Genet.* (e-pub ahead of print 10 September 2013; doi:10.1111/cge.12275).
10 Schilter, K. F., Reis, L. M., Schneider, A., Bardakjian, T. M., Abdul-Rahman, O., Kozel, B. A. *et al.* Whole-genome copy number variation analysis in anophthalmia and microphthalmia. *Clin. Genet.* **84,** 473–481 (2013).
11 Sato, N., Kamachi, Y., Kondoh, H., Shima, Y., Morohashi, K., Horikawa, R. *et al.* Hypogonadotropic hypogonadism in an adult female with a heterozygous hypomorphic mutation of SOX2. *Eur. J. Endocrinol.* **156,** 167–171 (2007).
12 Kelberman, D., de Castro, S. C., Huang, S., Crolla, J. A., Palmer, R., Gregory, J. W. *et al.* SOX2 plays a critical role in the pituitary, forebrain, and eye during human embryonic development. *J. Clin. Endocrinol. Metab.* **93,** 1865–1873 (2008).
13 Alatzoglou, K. S., Andoniadou, C. L., Kelberman, D., Buchanan, C. R., Crolla, J., Arriazu, M. C. *et al.* SOX2 haploinsufficiency is associated with slow progressing hypothalamo-pituitary tumours. *Hum. Mutat.* **32,** 1376–1380 (2011).
14 Schneider, A., Bardakjian, T., Reis, L. M., Tyler, R. C. & Semina, E. V. Novel SOX2 mutations and genotype-phenotype correlation in anophthalmia and microphthalmia. *Am. J. Med. Genet. A* **149A,** 2706–2715 (2009).
15 Li, J., Pan, G., Cui, K., Liu, Y., Xu, S. & Pei, D. A dominant-negative form of mouse SOX2 induces trophectoderm differentiation and progressive polyploidy in mouse embryonic stem cells. *J. Biol. Chem.* **282,** 19481–19492 (2007).
16 Salem, N. J., Hempel, M., Heiliger, K. J., Hosie, S., Meitinger, T. & Oexle, K. Anal atresia, coloboma, microphthalmia, and nasal skin tag in a female patient with 3.5 Mb deletion of 3q26 encompassing SOX2. *Am. J. Med. Genet. A* **161A,** 1421–1424 (2013).
17 Gu, W., Zhang, F. & Lupski, J. R. Mechanisms for human genomic rearrangements. *Pathogenetics* **1,** 4 (2008).
18 Chen, J. M., Cooper, D. N., Férec, C., Kehrer-Sawatzki, H. & Patrinos, G. P. Genomic rearrangements in inherited disease and cancer. *Semin. Cancer Biol.* **20,** 222–233 (2010).
19 Boone, P. M., Liu, P., Zhang, F., Carvalho, C. M., Towne, C. F., Batish, S. D. *et al.* Alu-specific microhomology-mediated deletion of the final exon of SPAST in three unrelated subjects with hereditary spastic paraplegia. *Genet. Med.* **13,** 582–592 (2011).
20 Fisher, E. & Scambler, P. Human haploinsufficiency—one for sorrow, two for joy. *Nat. Genet.* **7,** 5–7 (1994).

Supplementary Information accompanies the paper on Journal of Human Genetics website (http://www.nature.com/jhg)

J Clin Immunol (2014) 34:933–940
DOI 10.1007/s10875-014-0089-1

ORIGINAL RESEARCH

Interstitial Lung Disease with Multiple Micrograniulomas in Chronic Granulomatous Disease

Toshinao Kawai · Nobuyuki Watanabe · Midori Yokoyama · Yumiko Nakazawa · Fumihiro Goto · Toru Uchiyama · Masataka Higuchi · Takanobu Maekawa · Eiichiro Tamura · Satoshi Nagasaka · Masayuki Hojo · Masafumi Onodera

Received: 4 February 2014 / Accepted: 20 August 2014 / Published online: 4 September 2014

Abstract
Background Chronic granulomatous disease (CGD) is a primary immunodeficiency disease that is characterized by susceptibility to bacterial and fungal infections. CGD patients also suffer from immune regulatory disorders, such as CGD-associated bowel inflammation with granuloma, which could be caused by excessive inflammation without demonstrable infection.
Purpose We investigated the clinical manifestation of interstitial lung disease (ILD) resulting from excessive inflammation in X-linked CGD patients.
Methods Pulmonary CT images and testing of serum KL-6 levels were performed to assess ILD in the patients. For this study, patients with pulmonary lesions due to demonstrable infections were excluded from among ILD patients.
Results Among 33 CGD patients, four developed ILD; they had increased reticulo-nodular opacities on CT images and elevated serum KL-6 levels. Histopathological examinations revealed multiple homogeneous micrograniulomas in the lesions of inflammatory cell infiltration. Mononuclear cells obtained from their pulmonary lesions produced higher amounts of inflammatory cytokines than the peripheral blood mononuclear cells of CGD patients, suggesting that the only infiltrating cells in the pulmonary lesions were activated and produced large amounts of inflammatory cytokines in ILD patients. Interestingly, an anti-inflammatory drug, such as a corticosteroid or thalidomide, but not anti-bacterial or anti-fungal drugs, improved CT image findings and reduced their KL-6 levels.
Conclusions CGD patients' daily exposures to inhaled antigens may induce excessive reactions with the production of inflammatory cytokines leading to the development of ILD with multiple micrograniulomas, which could be due to an inadequate production of reactive oxygen species in CGD.

Keywords Chronic granulomatous disease · interstitial lung disease · inflammation · granuloma · hypersensitivity pneumonia

T. Kawai (✉) · N. Watanabe · M. Yokoyama · Y. Nakazawa · F. Goto · T. Uchiyama · M. Onodera
Department of Human Genetics, National Center for Child Health and Development, 2-10-1 Okura, Setagaya-ku, Tokyo 157-8535, Japan
e-mail: kawai-t@ncchd.go.jp

M. Higuchi
Division of Pulmonology, National Center for Child Health and Development, Tokyo, Japan

T. Maekawa
Department of Pediatrics, National Center for Child Health and Development, Tokyo, Japan

E. Tamura
Department of Pediatrics, Jikei University School of Medicine, Tokyo, Japan

S. Nagasaka
Division of General Thoracic Surgery, National Center for Global Health and Medicine Japan, Tokyo, Japan

M. Hojo
Division of Respiratory Medicine, National Center for Global Health and Medicine Japan, Tokyo, Japan

Introduction

Chronic granulomatous disease (CGD) is one of the primary immunodeficiency diseases that is characterized by inadequate production of reactive oxygen species (ROS) due to mutations of the genes that encode for the NADPH oxidase complex (NOX). As a result, CGD patients suffer from severe infections caused by pathogenic microorganisms, such as catalase-positive bacteria, mycobacterium, and fungi [1, 2].

Although progress in medical treatments, including new antibiotics and antifungal drugs, provides for infection control [3], other clinical manifestation of CGD has become problematic in these situations; namely, chronic hyperinflammation such as granuloma formation, CGD-associated bowel inflammation (CGD colitis) [4], and autoimmune disorders [5].

Although the mechanisms underlying this hyperinflammation are still under investigation, a plausible explanation is that the reduced ROS generated by impaired NOX function cannot adequately inhibit the production of inflammatory cytokines [6], and that this ROS deficit in CGD allows for the continuous production of inflammatory cytokines [7, 8], resulting in immune dysregulation or hyperinflammation. Thus, one of the effective therapeutic approaches for such hyperinflammation is the use of corticosteroids or immunosuppressive drugs [9].

Indeed, infliximab, a chimeric antibody against tumor necrosis factor-α (TNFα), has shown therapeutic efficacy for refractory CGD colitis, as TNFα is thought to play a critical role in granuloma formation in CGD. However, the use of these drugs increases a patient's susceptibility to pathogenic infections [10].

In this paper, we describe four patients with X-linked CGD who developed interstitial lung disease (ILD). These patients had elevated serum KL-6 levels and showed increased reticulo-nodular opacities on pulmonary CT images. Although their histopathological findings were reminiscent of those seen with hypersensitivity pneumonia (HP), treatment with allergen avoidance alone did not provide complete therapeutic effects for the clinical symptoms. On the other hand, anti-inflammatory drugs such as a corticosteroid or thalidomide did mitigate the clinical symptoms. Hence, ILD observed in these CGD patients was likely to be caused by excessive allergic reactions against non-pathogenic antigens.

Materials and Methods

Patients

All procedures and experiments were done after receiving informed consent from the patients or their parents. Our study protocol was approved by the Institutional Review Board of the National Center for Child Health and Development. In our hospital, there had been 33 patients with X-linked CGD confirmed by gene sequence analysis during the past 10 years. Four patients were confirmed to have developed ILD based on pulmonary CT images and elevated serum KL-6 level [11]. Among the ILD patients, those with pulmonary lesions due to demonstrable infections were excluded from this study.

Measurement of Specific IgG

Specific IgG antibody to *Aspergillus fumigatus* was determined in the serum of CGD patients (n=21; age, 19.0±10.1 year-old) including four ILD patients, and healthy volunteers (n=23; age, 17.6±9.1 year-old) using *A. fumigatus* IgG enzyme-linked immunosorbent assay kit (IBL, Hamburg, Germany).

Measurements of Cytokines in Serum and Bronchoalveolar Lavage Fluid

Serum levels of interleukin (IL)-6, IL-8, tumor necrosis factor-α (TNFα), and interferon-γ (IFNγ) were determined with a quantitative multiplex Milliplex system (Millipore, Billerica, MA) for CGD patients with ILD, X-linked CGD patients without demonstrable infections (n=10; age, 19.3±9.7 year-old), and healthy volunteers (n=10; age, 25.4±10.3 year-old). None of the CGD patients suffered from demonstrable infections. However, as they had previous pulmonary infections caused by bacteria or fungi, residual pathogens may remain due to elevated serum levels of βD-glucan (7.9±6.1 pg/ml; Normal range <10 pg/ml), thought to be a marker of fungal infection [12, 13]. Bronchoalveolar lavage (BAL) was performed during fiberoptic bronchoscopy under local anesthesia. Cytokine concentrations in BAL fluid (BALF) were also determined by Milliplex.

Lymphocyte Subset Analysis in Bronchoalveolar Lavage Fluid

Cells in BALF were characterized by flow cytometry (FACSAria; Becton, Dickinson and Company) using anti-human CD3, CD4, and CD8 monoclonal antibodies conjugated with allophycocyanin, phycoerythrin-Cy7, or peridinin–chlorophyll proteins-Cy5.5 (BioLegend, San Diego, CA).

Cytokine Production

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized whole blood of CGD patients by density gradient centrifugation. Infiltrating cells in the lung were obtained from centrifuging BALF. The concentrations of IL-6, IL-8, TNFα, and IFNγ were determined for 2×10^6 cells/ml PBMCs and lung infiltrating cells without any stimulation in RPMI containing penicillin/streptomycin and 5 % human serum (Sigma-Aldrich) [14]. Cells were incubated for 16 h and cytokines in culture supernatants were determined by Milliplex.