Molecular morphological and genetic study on the mechanisms of the failure of sex differentiation

三觜，友子

(Degree)
博士（農学）

(Date of Degree)
2010-03-25

(Date of Publication)
2010-03-31

(Resource Type)
doctoral thesis

(Report Number)
甲4948

(URL)
https://hdl.handle.net/20.500.14094/D1004948

Doctoral Dissertation

Molecular morphological and genetic study on the mechanisms of the failure of sex differentiation

January, 2010

Graduate School of Agricultural Science,

Kobe University

Mitsuhashi, Tomoko

Doctoral Dissertation

Molecular morphological and genetic study on the mechanisms of the failure of sex differentiation

性分化の破綻機構に関する分子形態遺伝学的研究

January, 2010

Graduate School of Agricultural Science,

Kobe University

Mitsuhashi, Tomoko

List of Contents

PREFACE

Sexual reproduction, which is a characteristic of most living creatures and results in increased genetic diversity, is a process through which the next generation inherits genetic information contained in the parental chromosomes. The essential purpose of sex determination or differentiation is to equip organisms with the necessary anatomy and physiology to allow sexual reproduction to occur. Sex determination or differentiation is thought to be extremely important and serve as the foundation of species preservation. The genetic sex of the mammalian embryos is established at fertilization, the point at which they inherit either an X or a Y chromosome from their father. The indifferent gonads develop into either testes or ovaries depending on their genotype, XY or XX, respectively. Further sexual development is then established by endocrine function of the gonads.

In 1947, Alfred Jost reported that if XX and XY rabbit fetuses were castrated in utero before sexual differentiation, they developed mullerian ducts and external genitalia of a female pattern. Therefore, it is assumed that sex determination or differentiation in mammals, including humans, does not operate according to individual male- and female-type programs, but rather according to a default female-type program, with the males being derived from the females. That is, the indifferent gonads differentiate into testes only when the Y chromosome exists, and automatically differentiate into ovaries when the Y chromosome is absent. Thus, XXY individuals develop testes [Jacobs and Strong, 1959] and XO individuals develop ovaries [Ford *et al*., 1959].

The sex-determining region of the Y chromosome (*SRY*) (GenBank accession number: NM_003140), which is located on the short arm of the Y chromosome, was identified in 1990 [Sinclair *et al*., 1990]. This testis-determining gene [Sinclair *et al*., 1990; Koopman *et al*., 1991] was shown to play a critical role in initiating testis

formation during gonadal development [Koopman *et al*., 1990, 1991]. In mice, *Sry* is first expressed around 10.5 days *post coitum* (dpc), shortly after the emergence of the genital ridges, its expression reaches peak levels at 11.5 dpc, and is extinguished by 12.5 dpc [Koopman *et al*., 1990; Hacker *et al*., 1995; Jeske *et al*., 1995]. In humans, *SRY* is transiently expressed during fetal development and reaches peak levels at 44 days of gestation, when the testis cords are first visible [Hanley *et al*., 2000]; and then its expression is vanishingly low. In other words, in mammals, sex determination or differentiation is chromosomally controlled by regulation of the *SRY* gene.

Identification of the *SRY* gene seemed to promise rapid elucidation of the mechanisms that underlie sex determination or differentiation, but it immediately became apparent that these processes are not controlled by a single gene. For example, many other genes, including SRY-box 9 (*SOX9*), fibroblast growth factor 9 (*FGF9*), and desert hedgehog homolog (Drosophila) (*DHH*), participate in the male sex-determination pathway [Wilhelm *et al*., 2007]. On the other hand, it is currently believed that sexual development in females (ovary differentiation) occurs by default; however, there is also genetic evidence of the existence of an "ovary organizer" (ovarian determinant, *Od*) [Eicher and Washburn, 1986] and "anti-testis" activity (termed *Z*) [McElreavey *et al*., 1993], which are thought to be necessary for ovary differentiation. It was also suggested that wingless-type MMTV integration site family, member 4 (*WNT4*), dosage-sensitive sex reversal adrenal hypoplasia congenita (AHC) critical region on the X chromosome, gene 1 (*DAX1*), and forkhead box L2 (*FOXL2*) probably participate in the early development of the ovary [Wilhelm *et al*., 2007]. In brief, it is not until these sex-determining genes are normally expressed in a spatio-temporal manner in the indifferent gonad that normal gonadal development is accomplished.

These sex-determining genes have been clarified following the development of new molecular biological techniques as well as through case analysis of patients with

disorders of sex development (DSD). The term DSD encompasses different biological status of sexual differentiation at any level, from the chromosomal to the hormonal. Various types of DSD are known to occur in various mammalian species. For example, DSD have been reported to occur in roe deer (*Capreolus capreolus*) [Pajares *et al*., 2009], goats (*Capra hircus*), llama (*Llama glama*), sheep (*Ovis aries*), pigs (*Sus scrofa*), lesser pandas (*Ailurus fulgens*), dogs (*Canis familiaris*), cats (*Felis cattus*), raccoon dogs (*Nyctereutes procyonoides*), belugas (*Delphinapterus leucas*), moles (*Talpa sp*.), rabbits (*Oryctolagus cuniculus*), grey kangaroos (*Macropus giganteus*), agile wallabies (*Macropus agilis*), American opossums (*Monodelphis domestica*), horses (*Equus caballus*), grass mice (*Akodon sp*.), voles (*Microtus cabrerae*), mice (*Mus musculus*), wood lemmings (*Myopus schisticolor*), and humans (*Homo sapiens*) [reviewed by Vaiman and Pailhoux, 2000]. DSD are thus far from rare and are especially prevalent in humans. Disorders of sex determination or differentiation in humans lead to malformations of the internal and/or external genitalia (resulting in an XY female, XX hermaphrodite, and XX male or pseudohermaphrodite). DSD occur at a frequency of 1 in 2,000 or in approximately 0.2–0.3% of the population [Fujimoto and Hayashi, 1983]. The pathophysiology of DSD is complex and includes numerical/structural aberrations in sex chromosomes, mutations in genes associated with sex differentiation, disorders in the formation/action of steroid hormones, and defects in androgen receptors and other factors.

XY gonadal dysgenesis, first described in 1955 by Swyer, is estimated to occur at a frequency of 1:20,000 [Robinson and Linden, 1993]. Individuals with this condition appear to be normal females at birth, but they do not develop secondary sexual characteristics at puberty, do not menstruate, and develop streak gonads. Mutations in *SRY* are associated with XY females. The following types of mutations have been detected in the *SRY* gene: missense [Hawkins *et al*., 1992a; Zeng *et al*., 1993; Imai *et al*.,

1999; Okuhara *et al*., 2000; Shahid *et al*., 2004], nonsense [Hawkins *et al*., 1992a; Müller *et al*., 1992; Iida *et al*., 1994; Giuffrè *et al*., 2004], and frameshift mutations [Jäger *et al*., 1990; Kellermayer *et al*., 2005]. However, *SRY* mutations are found in approximately 10–20% of XY females [Hawkins *et al*., 1992b; Lim *et al*., 1998]; this suggests a need for a new approach to understand the underlying mechanisms of DSD.

Recently, it was suggested that gene expression is regulated via an epigenetic mechanism. Epigenetic changes induce persistently altered gene expression in the absence of gene mutations. In higher vertebrates, DNA methylation generally occurs at the cytosine residue of CpG dinucleotides, and 60–70% CpGs are methylated [Gruenbaum *et al*., 1981; Razin *et al*., 1984; Boyes and Bird, 1992]. Recently, it was reported that in mice, *Sry* gene expression is epigenetically regulated by DNA methylation-mediated gene silencing [Nishino *et al*., 2004]. In clinical cases, as a result of a pericentric inversion, *SRY* is inverted to the middle of the heterochromatic region. CpG methylation-mediated positional effect variegation (PEV) leads to *SRY* gene silencing, resulting in the XY female [Gimelli *et al*., 2006]. Furthermore, core histones can be reversibly modified by acetylation, methylation, phosphorylation, ubiquitination, or adenosine diphosphate (ADP) ribosylation. Modification of histones and remodeling of chromatin structures play critical roles in the regulation of gene transcription and chromosome replication [Strahl and Allis, 2000; Ng and Bird, 2000; Grunstein, 1997; Kadonaga, 1998]. Epigenetic alteration of *SRY* seems to be one of the wide-ranging pathogenetic mechanisms in the sex differentiation anomaly.

Meanwhile, studying an XY female with no detectable mutations in the *SRY* gene may be relevant to understanding alterations in sex-determining genes located downstream of *SRY* and/or in genes located on the X chromosome or autosomes (including not only genes related to sex differentiation but also genes related to the entire genome). Recently, the developments of both DNA microarray techniques and

bioinformatics have enabled analysis of the expression of several tens of thousands of genes simultaneously, and novel biological interpretations have been made by cross-checking existing databases. Given the complexity of DSD and our limited understanding of its underlying pathogenetic mechanisms, it would be useful to exhaustively analyze changes in the gene expression profiles between a normal adult male and a DSD patient. In addition, it would be very useful to translate the information on individual genes into a broader characterization by using systematic functional annotation such as Gene Ontology (GO) analysis [Ashburner *et al*., 2000] and Gene Microarray Pathway Profiler (GenMAPP) [Dahlquist *et al*., 2002]. From a collective and global perspective, analysis of changes in the expressions of individual genes could provide an understanding of the differences that exist at the molecular level between normal adult males and DSD patients.

Many unexplained phenomena have been encountered in clinical experience [Hoshi *et al*., 1998a, b], and it might not be too much to say that even today, 20 years after the *SRY* gene was identified [Sinclair *et al*., 1990], investigators are still searching through the clues to fully understand sexual determination and differentiation. In the present study, I examined an idiopathic XY female by cytogenetic, histological, and molecular analyses, including comprehensive bioinformatic analysis. In particular, emphasis was laid on the association between the regulatory mechanism of *SRY* gene expression and epigenetic alteration, and on the mutual interaction or coordinated action between altered genes. In the final analyses, I attempted to reveal the changes occurring at the molecular level that contribute to DSD and propose a novel mechanism to explain disorders of sex differentiation in males. A study on the functional relationship between related genes would provide novel perspectives on the molecular basis of DSD. My results provide some insights into the functioning mechanisms leading to DSD.

Chapter I:

Abnormally prolonged *SRY* expression in the streak gonads and skin of an adult XY female patient

INTRODUCTION

Disorders of human sex determination or differentiation trigger malformations of the internal and/or external genitalia, ranging from genital ambiguity to complete sex reversal such as the XY female and the XX male. DSD are not rare; they occur in approximately 0.2–0.3% of the population [Fujimoto and Hayashi, 1983]. XY females carry a Y chromosome but are phenotypically female. At birth, patients with the XY female type of gonadal dysgenesis (Swyer syndrome) appear to be normal; however, they do not develop secondary sexual characteristics at puberty, do not menstruate, and have streak gonads. The incidence of Swyer syndrome is estimated at 1:20,000 [Robinson and Linden, 1993]. Mutations in *SRY*, a testis-determining gene [Sinclair *et al*., 1990; Koopman *et al*., 1991], are associated with XY females. These mutations, in most cases within the high mobility group (HMG) box, reportedly affect the gene's DNA-binding [Hawkins *et al*., 1992b], DNA-bending [Pontiggia *et al*., 1994], and nuclear-localization activities [Li *et al*., 2001], resulting in DSD. However, *SRY* mutations are found in approximately 10–20% of the XY females [Hawkins *et al*., 1992b; Lim *et al*., 1998], suggesting that other factors influence sex differentiation.

SRY is located on the short arm of the Y chromosome and plays a critical role in initiating testis formation during gonadal development [Koopman *et al*., 1990, 1991]. Although it was identified in 1990 [Sinclair *et al*., 1990], its mode of action remains unknown. In humans, *SRY* mRNA is detectable at 41 days of gestation in XY embryos, and the *SRY* levels peak at 44 days of gestation, when the testis cords are first visible [Hanley *et al*., 2000]. By 52 days of gestation, the germ cells are surrounded by Sertoli cells, which continue to express *SRY* mRNA at a low level [Hanley *et al*., 2000]. Other factors, including the genes *SOX9*, *FGF9*, and *DHH*, are also concerned with the male sex-determination pathway. However, some mutations are not found in those genes, and

the cause of DSD is not simple. In addition, many unexplained phenomena have been encountered in clinical practice.

Thus far, numerous studies on XY females have been conducted from the perspective of point mutations [Biason-Lauber *et al*., 2009; Cost *et al*., 2009; Marchina *et al*., 2009] or deletions [Smyk *et al*., 2007; van Silfhout *et al*., 2009] in the sex-determining genes, disorder of steroid formation or action [Radpour *et al*., 2009], sex chromosomal disorder [Piccione *et al*., 2008], and histopathological characteristics of an isolated gonad [Salas-Cortés *et al*., 2000]. However, the expression of the sex-determining genes including *SRY* in both the classical organ developing through the sex-determining genes (gonad) and the peripheral tissue (skin) of adult XY females is unknown. In this study, I examined gonadal tissue and fibroblasts derived from a 17-year-old woman suspected of having DSD by cytogenetic, histological, and molecular analyses, with particular emphasis on the sex-determining gene expressions.

MATERIALS AND METHODS

Written informed consent was obtained from the patient and her parents. The study was approved by the institutional ethical board of Hokkaido University Graduate School of Medicine.

Subject

The patient, a 17-year-old Japanese woman (gravida 0, para 0) of normal height, was born to healthy parents as a low-birth-weight infant (1,910 g) with normal female external genitalia at 39 weeks of gestation. She was referred to our institution for primary amenorrhea with lack of pubic and axillary hair as well as underdeveloped breasts. Pelvic magnetic resonance imaging (MRI) disclosed a uterus of normal size and shape, which appeared to be functional based on withdrawal bleeding in response to administration of hormonal agents; ovaries were not identifiable, and there were no signs of a mass on either side of the inguinal region. She had elevated gonadotropin levels (LH = 117.1 mIU/mL, FSH = 417.5 mIU/mL) and extremely low levels of steroid hormones (testosterone = 0.22 ng/mL, progesterone = 0.35 ng/mL, estradiol (E2) = undetectable). Her medical history was notable for membranous nephropathy and an inguinal hernia requiring surgical repair at age two.

Cell Culture

Primary cultures were established from the gonads and the skin of the elbows. A human male cell line from skin fibroblasts of a normal young man was purchased from Cell Systems (Kirkland, WA, USA) and used as a normal male control. The cells were cultured in Minimum Essential Medium α (α-MEM; Invitrogen-Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Biowest, Nuaillé, France) and

incubated in 25 cm^2 cell culture flasks at 37°C in a humidified atmosphere with 5% CO_2. The medium was changed regularly, and the cells were grown to confluence to standardize the state of proliferation as much as possible.

Cytogenetics

Gonadal tissue, peripheral blood, and fibroblasts were cultured by routine techniques and used in repeated chromosome analyses by the G- and Q-banding methods with Leica CW4000 (Leica Microsystems Imaging Solutions Ltd., Cambridge, UK).

Fluorescent *In Situ* Hybridization (FISH)

FISH was performed to confirm the localization of *SRY* in the pericentrically inverted chromosome. Hybridization of probes to chromosomal DNA from the patient was performed as described previously [Hoshi *et al*., 1998b]. The probes (Vysis, Inc., Downers Grove, IL, USA) used in this study included SRY, alpha satellite Y-centromere locus DYZ3, and Y-long arm terminal portion (Yq12)-specific DYZ1.

DNA and RNA Extraction

Genomic DNA was extracted from cultured cells (AllPrep DNA/RNA mini kit; Qiagen, Hilden, Germany) and peripheral blood (Wizard genomic DNA purification kit; Promega, Madison, WI, USA). Total cellular RNA was extracted from cultured cells using an AllPrep DNA/RNA mini kit (Qiagen) and treated with RNase-free DNase to eliminate genomic DNA contamination.

DNA Analysis

For the peripheral blood, fibroblast, and gonadal DNA, I used a set of oligoprimers representing eight different loci (*PABY*, *SRY*, *AMGL*, *DYZ3*, *DYS139*, *DYS132*, *DYS1*,

and *DYZ1*) of the Y chromosome from the distal short arm to the distal long arm for PCR amplifications with GoTaq Flexi DNA polymerase (Promega). The primer sequences are listed in Table I-1. The thermal cycling conditions for this amplification were as follows: initial denaturation at 94°C for 8 min; 33 cycles of denaturation at 94°C for 1 min, annealing at 57°C for 1 min, and extension at 72°C for 1 min; and final extension at 72°C for 7 min. The PCR products were separated by electrophoresis on 2% agarose gel containing ethidium bromide.

Mutation Analysis

I analyzed the *SRY* sequence in the gonadal tissue and fibroblasts. The DNA was amplified by PCR and specific primers, the sequences of which are listed in Table I-1. The PCR conditions for this amplification were as follows: initial denaturation at 95°C for 5 min; 30 cycles of denaturation at 95°C for 30 sec, annealing at 65°C for 30 sec, and extension at 72°C for 1 min; and final extension at 72ºC for 7 min. The PCR products were sequenced with the Taq DyeDeoxy sequencing regents and analyzed on an ABI Prism 3100 genetic analyzer (Applied Biosystems, Foster City, CA, USA) [Sugawara *et al*., 1995].

Histological Analysis of Gonadal Tissue

Gonadal tissue from the patient was fixed in 10% neutral buffered formalin for 24 hr, and processed tissue was embedded in paraffin. Sections were cut at 4 μm thickness and stained with hematoxylin and eosin (H&E; Merck & Co. Inc., Whitehouse Station, NJ, USA) for general histological analysis.

Immunohistochemical Analysis of Gonadal Tissue

For the immunohistochemical analysis, deparaffinized gonadal sections were

autoclaved at 121°C for 20 min in 10 mM sodium citrate buffer (pH 6.0) for antigen retrieval, washed in 10 mM PBS, and then treated with 3% H_2O_2 dissolved in 100% methanol to inactivate endogenous peroxidase activity. The primary antibody for SRY (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) was diluted at ratios of 1:100 in 10 mM PBS and incubated over the sections in a humidified chamber for 14 hr at 4°C. The immunohistochemical analysis for SRY was conducted using DakoCytomation EnVision$^+$ System-HRP Labeled Polymer (Dako, Glostrup, Denmark). The immunoreactions were visualized with 3,3′-diaminobenzidine solution (Dako) containing 0.03% H_2O_2 and light counterstaining with Mayer's hematoxylin.

Qualitative and Quantitative RT-PCR

One microgram of total RNA was reverse-transcribed to cDNA using a Super Script First-Strand Synthesis System (Invitrogen).

The expression of *SRY* mRNA was examined in gonadal tissue by qualitative RT-PCR. The cDNA was amplified by PCR with the specific primer set. The primer sequence for *SRY* is listed in Table I-2. The thermocycling program for this amplification was as follows: initial denaturation at 95°C for 5 min; 36 cycles of denaturation at 95°C for 30 sec, annealing at 65°C for 30 sec, and extension at 72°C for 1 min; and final extension at 72°C for 7 min. The PCR products were separated by electrophoresis on 2% agarose gel containing ethidium bromide.

The mRNA expressions of *SRY*, *SOX9*, *FGF9*, *WNT4*, Wilms tumor 1 (*WT1*), *DAX1*, GATA binding protein 4 (*GATA4*), Müllerian-inhibiting hormone (*MIS*), *DHH*, steroidogenic factor 1 (*SF1*), androgen receptor (*AR*), estrogen receptor α (*ERα*), and p300/CBP-associated factor (*PCAF*) were examined in fibroblasts by quantitative RT-PCR. The cDNA was amplified by PCR with the specific primer set (Table I-2). Real-time PCR was performed by using a LightCycler rapid thermal cycler system

(Roche Diagnostics Ltd., Lewes, UK) with LightCycler FastStart DNA Master^{PLUS} SYBR Green I mix (Roche Diagnostics Ltd.). The amplification protocol consisted of one cycle at 95°C for 10 min followed by the appropriate number of cycles of 95°C for 10 sec, 60°C for 5 sec, and 72°C for 20 sec. Amplification of glyceraldehyde-3'-phosphate dehydrogenase (*GAPDH*) mRNA was used as an internal positive control, and the amounts of each mRNA were normalized to the *GAPDH* mRNA levels in each sample. The PCR results were analyzed by using the $2^{-\Delta\Delta CT}$ method [Livak and Schmittgen, 2001].

Chromatin Immunoprecipitation (ChIP) Assay

To examine the acetylation status in the *SRY* region of the fibroblasts, ChIP was performed by using a ChIP assay kit (Upstate Cell Signaling Solutions, Charlottesville, VA, USA). In brief, DNA was cross-linked to the associated proteins with formaldehyde, followed by sonication. Immunoprecipitations were carried out with anti-acetylated histone H3 antibodies (3 μg). The formaldehyde cross-links were then reversed, and DNA was purified by phenol/chloroform extraction and ethanol precipitation. PCR was performed to amplify the DNA bound to the immunoprecipitated histone by using the primers listed in Table I-1. I used Primer3 (Massachusetts Institute of Technology) to design the specific primers. Input DNA, before immunoprecipitation, was also subjected to PCR as a control. The thermal profile for this amplification was as follows: initial denaturation at 95°C for 5 min; 30 cycles of denaturation at 94°C for 30 sec, annealing at 57°C for 30 sec, and extension at 72°C for 1 min; and final extension at 72°C for 7 min. The PCR products were separated by electrophoresis on 2% agarose gel containing ethidium bromide. The intensity of the ethidium bromide luminescence was measured with the CS Analyzer software (ATTO Corporation, Tokyo, Japan).

Microarray Analysis

For gene expression analysis of the fibroblasts, I used a GeneChip system with Human Expression Array U133A spotted with 22,283 probe sets (Affymetrix, Santa Clara, CA, USA). Sample preparation for the array hybridization was carried out, and biotin-labeled cRNA probes were generated from 5 μg of DNase-treated total RNA. After fragmentation, the biotinylated cRNA probe was hybridized to arrays at 45°C for 16 hr. The arrays were washed, stained with streptavidin–phycoerythrin, and scanned with a probe array scanner. The scanned chip was analyzed by using the GeneChip Analysis Suite software (Affymetrix). The hybridization intensity data were converted into a presence or absence call for each gene, and changes in gene expression between the experiments were detected by comparison analysis. The data were further analyzed by using the GeneSpring software (Agilent, Santa Clara, CA, USA) to extract the significant genes [Tabuchi *et al*., 2006].

Statistical Analysis

The values are presented as the mean ± SE. Statistical analyses were performed with StatView for Windows (version 5.0; SAS Institute, Inc., Cary, NC, USA), using Student's *t*-test, and *p* values less than 0.05 were regarded as indicating a significant difference.

RESULTS

Karyotyping

Chromosomal examinations of the patient's gonadal tissue, peripheral blood, and fibroblasts revealed a male 46,XY karyotype, and the short arm of the Y chromosome was longer than that of a normal Y chromosome. The G- and Q-banding revealed the structural anomalies. An atypical G-banding-positive chromosomal segment was observed in the short arm of the Y chromosome (Fig. I-1A), and the Q-banded karyotype of the Y chromosome showed brilliant fluorescence only in the long arm (q12), not the short arm (Fig. I-1B). Thus, the patient's Y chromosome was inv(Y)(p11.2q11.2) (Fig. I-1E).

FISH Findings

Dual-color FISH analysis of the metaphase chromosome revealed that two signals (DYZ3 and DYZ1) were located nearby (Fig. I-1C) and that two signals (SRY and DYZ1) were completely separated and in their specific positions (Fig. I-1D). Therefore, *SRY* still remained in the distal portion of the short arm of the Y chromosome.

Deletion Analysis

The eight different loci (*PABY*, *SRY*, *AMGL*, *DYZ3*, *DYS139*, *DYS132*, *DYS1*, and *DYZ1*) of the Y chromosome from the distal short arm to the distal long arm were all present (Table I-3). No large segments of the Y chromosome were deleted in the samples from peripheral blood, fibroblasts, and gonadal tissue.

SRY Sequencing

DNA sequence analysis of *SRY* in the fibroblasts and gonads disclosed no substantial

changes from the reference sequence. A previously identified synonymous single nucleotide polymorphism (SNP) (rs11575897. c.465C>T; p.S155S) was found in homozygous form (Fig. I-1F). This SNP is described in dbSNP (http://www.ncbi.nlm.nih.gov/SNP/) as occurring at position 613 in *SRY* mRNA. No other mutation associated with functional abnormality was detected.

Histological Findings of Gonadal Tissue

The patient's gonadal tissues consisted of fibrous stroma without germ cells and neither a testicular nor an ovarian differentiation pattern (Fig. I-2A). Histological review of the gonadal tissues yielded a diagnosis of streak gonad, including Leydig-like cells with eosinophilic cytoplasm (Fig. I-2B) and formation of duct-like structures (Fig. I-2C). This was undifferentiated gonadal tissue that had lost its germ cells and undergone fibromatous involution.

***SRY* mRNA Expression and Immunohistochemical Findings in the Streak Gonads**

RT-PCR revealed *SRY* mRNA as a 270-bp band (Fig. I-3A). The mRNA was expressed in the streak gonads. SRY immunoexpression was detected in the irregularly shaped nucleus of parenchymal cells in the streak gonadal tissue (Fig. I-3B), which also had SRY-negative cells. SRY was strongly expressed throughout the streak gonads, mostly in the fibrous stroma (Fig. I-3B). The negative control sections treated with nonimmunized rabbit sera in place of the primary antibody did not show nonspecific staining (data not shown).

Expression of Sex-determining Genes in the Fibroblasts

The expressions of *SRY* ($p < 0.01$), *WNT4* ($p < 0.0001$), and *WT1* ($p < 0.001$) mRNA were scarcely detected in the normal male fibroblasts (Fig. I-4A). The levels of *DHH* (*p*

< 0.01), *SF1* ($p < 0.0001$), and *ERα* ($p < 0.005$) in the fibroblasts from the patient were significantly higher than those in the normal male control. In contrast, the levels of *SOX9*, *FGF9*, and *AR* mRNA expression in the patient were significantly lower than those in the normal male ($p < 0.05$). *GATA4* mRNA expression in the patient was scarcely detected ($p < 0.05$). There was no clear difference in the expression levels of *MIS* and *DAX1* mRNAs between the patient and the normal male; *MIS* tended to be lower in the patient ($p = 0.0677$).

Histone H3 Acetylation by ChIP Assay

Histone H3 in the *SRY* region was acetylated in both the fibroblasts from the patient and those derived from the normal male control (Fig. I-4B). However, histone H3 of *SRY* in the fibroblasts from the patient was significantly hyperacetylated ($p = 0.0021$).

Gene Expression Analysis by Microarrays

I identified 1,366 probe sets (786 upregulated and 580 downregulated) showing significant differences in transcription levels between the patient and the normal male, revealing striking differences in the gene expression profiles (data not shown). The gene expressions on autosomal chromosomes were altered remarkably relative to those on the Y chromosome with pericentric inversion. Of these altered probe sets, a change in the expression level of *PCAF* was included as a possible candidate involved in the histone hyperacetylation of *SRY*. Other factors that can acetylate the histone protein were not detected. Real-time RT-PCR (n = 3) of *PCAF* revealed a 3.7-fold higher level in this patient than in the normal male control (Fig. I-4A). The trend in expression level of *PCAF* gene detected by real-time RT-PCR was consistent with the result obtained from microarrays.

DISCUSSION

The prevalence of males with pericentric inversion of the Y chromosome that does not influence the phenotype is approximately 1:1000 in the general population [Zeuthen and Nielsen, 1973; Tóth *et al*., 1984; Schmid 1985; Krishna Murthy *et al*., 1989]. In contrast, there are reported instances in which pericentric inversion of the Y chromosome caused XY females: if *SRY* is inverted and relocated to the center of the heterochromatic region (Yq12), this positional variegation will lead to *SRY* gene silencing, inducing XY female development [Gimelli *et al*., 2006]. Detailed cytogenetic analysis using repeated chromosomal examination by the G- and Q-banding methods with the gonadal tissue, peripheral blood, and fibroblasts revealed that this patient had the 46,X,inv(Y)(p11.2q11.2) karyotype, and no large segments of the Y chromosome were deleted, showing a whole Y chromosome. In addition, *SRY* was not inverted and relocated to the heterochromatic region but rather was located in the original position (Yp11.3). Therefore, the previously reported case does not apply to the patient. This study describes the first gene expression analysis using both the gonadal tissue and skin of an adult XY female with pericentric inversion of the Y chromosome but without functional mutation in *SRY*. I conducted the study to reveal the possible molecular context contributing to this condition.

In the streak gonads of this patient, Leydig-like cells with eosinophilic cytoplasm and formation of duct-like structures were detected. Furthermore, there were strongly SRY-immunopositive cells in a mostly fibrous stroma. Primary cultures established from the gonadal biopsies of this patient obviously expressed *SRY* mRNA, suggesting that *SRY* expression was still in progress in the streak gonads. Spermatogonia, round spermatids, and Sertoli cells of adult testes reportedly express *SRY* [Clépet *et al*., 1993; Olesen *et al*., 2001; Salas-Cortés *et al*, 1999, 2001; Modi *et al*., 2005]; however, in the

streak gonads of this patient, the SRY-immunopositive cells were evidently not those cells. SRY immunoexpression in streak gonads showing 46,XY gonadal dysgenesis without mutation in *SRY* has been reported by Salas-Cortés *et al*. [2000], and my findings support those of the previous study. In humans, the *SRY* mRNA levels essentially peak at 44 days of gestation, when the testis cords are first visible, and are then continuously expressed at a low level [Hanley *et al*., 2000]. The finding of an XY female with prolonged *SRY* expression in the streak gonads implies that appropriate regulation of *SRY* expression was impaired.

RT-PCR performed with total RNA extracted from the fibroblasts revealed that *SRY* mRNA was significantly overexpressed, relative to the normal male, which is an unexpected result because such high expression is seen only in the genital primordium during the fetal period [Hanley *et al*., 2000]. I consider that the difference in the *SRY* mRNA expression levels between this patient and the normal male may hold important clues to understanding the genetic pathogenesis of DSD. I focused my attention on chromatin modification as a possible explanation for the increased *SRY* expression. Chromatin modification is one of the principal methods of gene-expression regulation. In general, histone H3 acetylation is concerned with the activation of transcription [Wolffe and Pruss, 1996; Grunstein, 1997]. ChIP assay is used to analyze chromatin in its native environment in living cells, increasing our understanding of the impact of epigenetic modifications of nucleosomal histones on transcriptional regulation. Relative to the normal male control, the acetylated histone levels in the patient's fibroblasts were high in the *SRY* region. *SRY* mRNA overexpression was attributable to histone hyperacetylation. To my knowledge, this is the first case of DSD associated with *SRY* mRNA overexpression and highly acetylated histones.

To explore the possible etiologies of these results, I performed gene expression profiling studies to characterize the genetic differences broadly between this patient and

the normal male, and searched for associated candidate genes that can induce histone H3 acetylation. Interestingly, a change in the expression level of *PCAF*, a transcriptional coactivator with intrinsic histone acetyltransferase activity [Yang *et al*., 1996], was included as a possible candidate; other factors that can acetylate the histone protein were not detected. Real-time RT-PCR revealed a 3.7-fold higher level of *PCAF* expression in this patient than in the normal male control; the elevated levels suggest a possible mechanism for the activation of *SRY* expression via histone H3 acetylation.

Unexpectedly, the expressions of sex-differentiation genes such as *SOX9*, *FGF9*, *WNT4*, *WT1*, *GATA4*, *DHH*, *SF1*, *AR*, and *ERα* were significantly changed in the fibroblasts of this patient relative to those of the normal male. *SRY* is epistatic to these genes in the sex-determination pathway, and prolonged high *SRY* mRNA expression induces a destabilizing effect on the expressions of the downstream sex-determining genes. In fact, the other genes related to sex differentiation—prolactin receptor (*PRLR*), activin A receptor type IIA (*ACVR2A*), fibroblast growth factor 2 (*FGF2*), homeo box A10 (*HOXA10*), homeo box A11 (*HOXA11*), and bone morphogenetic protein 8b (*BMP-8B*)—were also detected by the microarray analysis as the genes with significantly changed expressions in comparison with those of the normal male (data not shown). In the streak gonads, which continue to express *SRY* mRNA, a similar phenomenon may be suggested. The correct regulation of *SRY* mRNA expression is essential for normal sex differentiation, and a disturbance might inhibit diversion into the masculinization cascade, thereby tilting the sexual fate toward the feminization cascade, the default pathway in mammals, even if SRY is translated normally. The findings in this study provide an opportunity to understand the importance of appropriate regulation of *SRY* expression in humans. Further investigation is required to evaluate the relationship between prolonged high *SRY* expression and DSD by a reconstitution experiment using laboratory animal models.

In conclusion, the sex-determining genes were expressed not only in the gonads but also in the skin of the adult 46,XY female patient as a vestige of sex differentiation anomalies in the fetal period. In normal ontogenetic development, *SRY* expression, involved in the first step of the male sex differentiation (depending on coordinated expression of specific genes), is regulated in a strictly temporal and spatial manner. This patient with prolonged activity of *SRY* provides a new insight into DSD and may help to reveal the cause of human XY gonadal dysgenesis.

SUMMARY

In normal ontogenetic development, the expression of the *SRY* gene, involved in the first step of male sex differentiation, is spatiotemporally regulated in an elaborate fashion. *SRY* is expressed in germ cells and Sertoli cells in adult testes. However, only few reports have focused on the expressions of *SRY* and the other sex-determining genes in both the classical organ developing through these genes (gonad) and the peripheral tissue (skin) of adult XY females. In this study, I examined the gonadal tissue and fibroblasts of a 17-year-old woman suspected of having disorders of sexual differentiation by cytogenetic, histological, and molecular analyses. The patient was found to have the 46,X,inv(Y)(p11.2q11.2) karyotype and streak gonads with abnormally prolonged *SRY* expression. The sex-determining gene expressions in the patient-derived fibroblasts were significantly changed relative to those from a normal male. Furthermore, the acetylated histone H3 levels in the *SRY* region were significantly high relative to those of the normal male. As *SRY* is epistatic in the sex-determination pathway, the prolonged *SRY* expression possibly induced a destabilizing effect on the expressions of the downstream sex-determining genes. Collectively, alterations in the sex-determining gene expressions persisted because of disorders of sexual differentiation not only in the streak gonads but also in the skin of the patient. The findings suggest that correct regulation of *SRY* expression is crucial for normal male sex differentiation, even if SRY is translated normally.

FIGURES AND TABLES

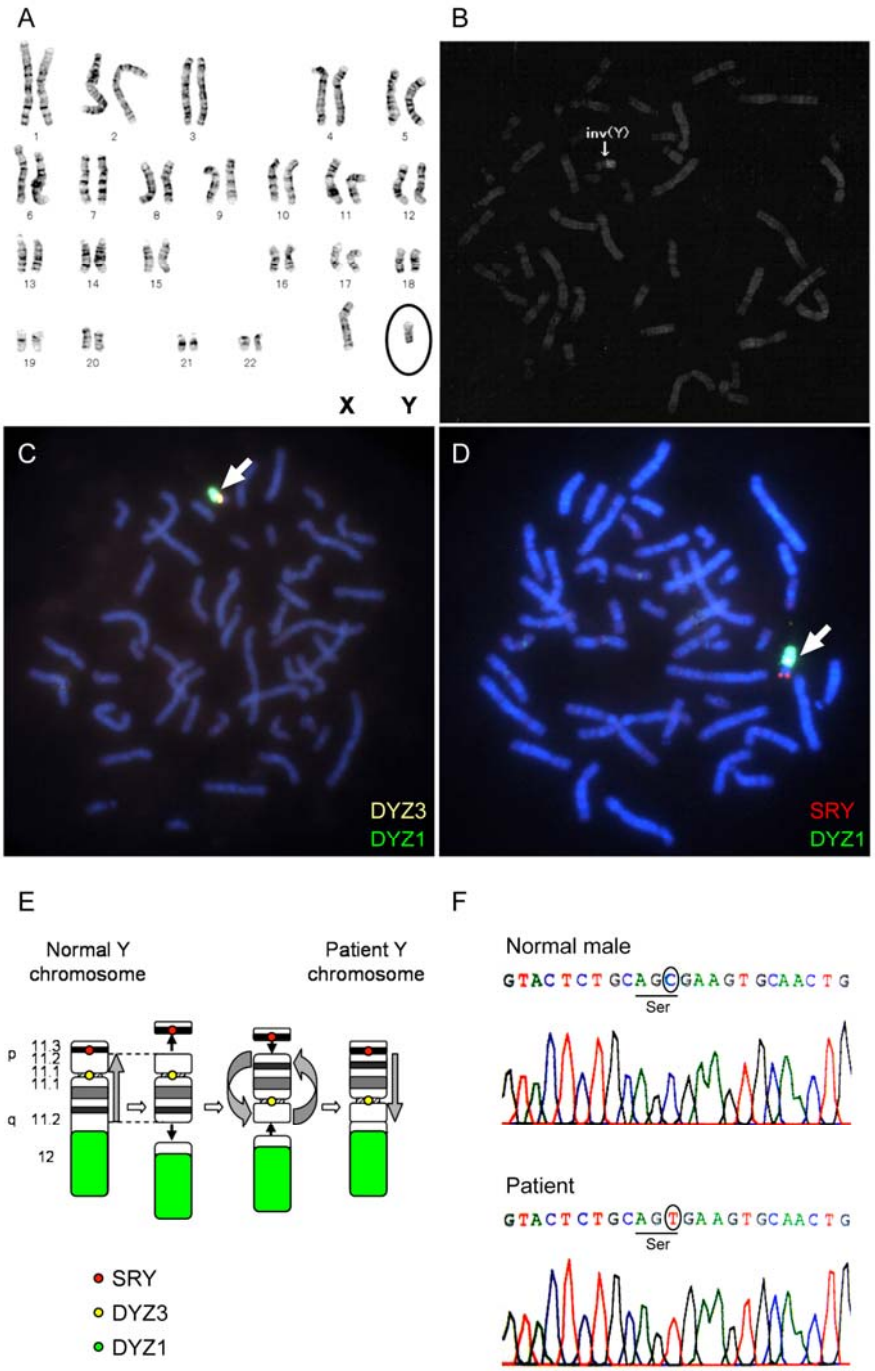

Fig. I-1

(**A**) Metaphase of cultured gonadal tissue and peripheral blood from the patient by G-banding. An atypical G-banding-positive chromosomal segment was observed in the short arm of the Y chromosome. The circle indicates the Y chromosome with pericentric inversion. (**B**) Metaphase of cultured gonadal tissue and peripheral blood from the patient by Q-banding. The Q-banded karyotype of the Y chromosome showed brilliant fluorescence in the long arm (q12). (**C**) Metaphase FISH with the DYZ3 and DYZ1 probes. Dual-color FISH (arrow) analysis of the metaphase chromosome revealed that two signals (DYZ3, centromere of the Y chromosome, yellow; DYZ1, Y-long arm terminal portion [Yq12], green) were located nearby. (**D**) Metaphase FISH with the SRY and DYZ1 probes. Dual-color FISH analysis using an SRY-specific probe (red) and a DYZ1-specific probe (green) showed that the two signals were completely separated and in their specific positions (arrow). (**E**) Schematic of the mechanism of Y chromosome pericentric inversion —46,X,inv(Y)(p11.2q11.2). (**F**) DNA sequencing of *SRY* in the gonadal tissue and fibroblasts. A previously identified synonymous SNP (rs11575897. c.465C>T; p.S155S) was found in homozygous form and is described in dbSNP (http://www. ncbi.nlm.nih.gov/SNP/) as occurring at position 613 in *SRY* mRNA.

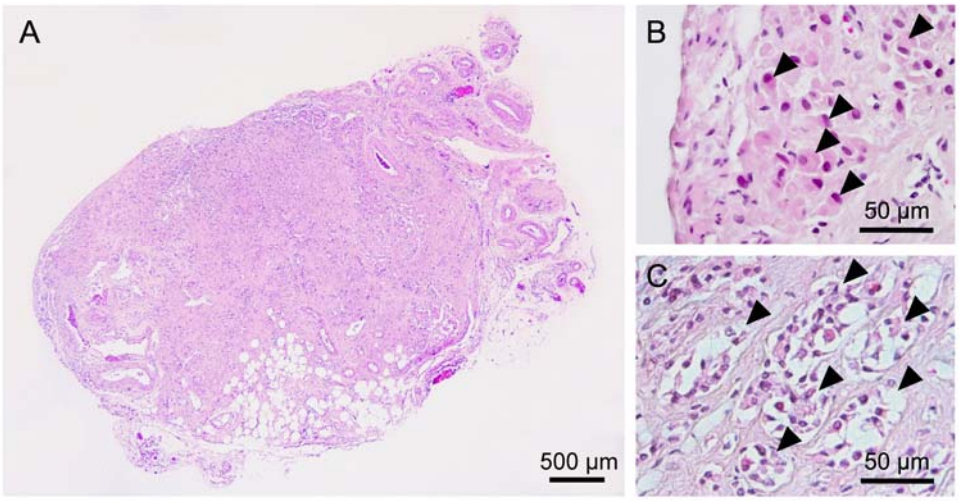

Fig. I-2

(**A**) Representative histological image of the gonadal tissue from the patient showing fibrous stroma without germ cells and neither a testicular nor an ovarian differentiation pattern. (**B**) Leydig-like cells with eosinophilic cytoplasm (arrowheads) were recognizable. (**C**) Formation of duct-like structures (arrowheads) was also recognizable. This was undifferentiated gonadal tissue that had lost its germ cells and undergone fibromatous involution. The histological investigation of the gonadal tissues yielded a diagnosis of streak gonads.

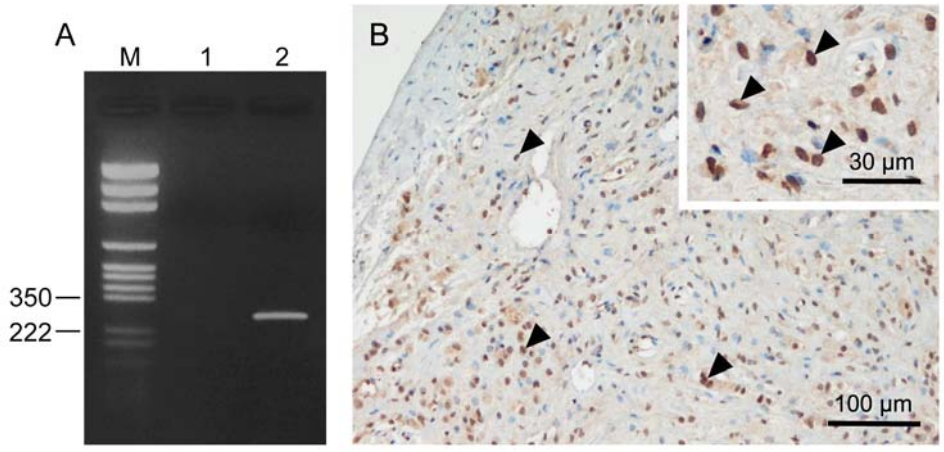

Fig. I-3

(**A**) Qualitative RT-PCR analysis of *SRY* mRNA expression (at 270 bp) in the streak gonads. M, size marker; lane 1, negative control (–DNA); lane 2, the streak gonadal tissue. (**B**) SRY expression found in the parenchymal cells of the patient's streak gonads (arrowheads). The inset shows a higher magnification of the parenchymal cells.

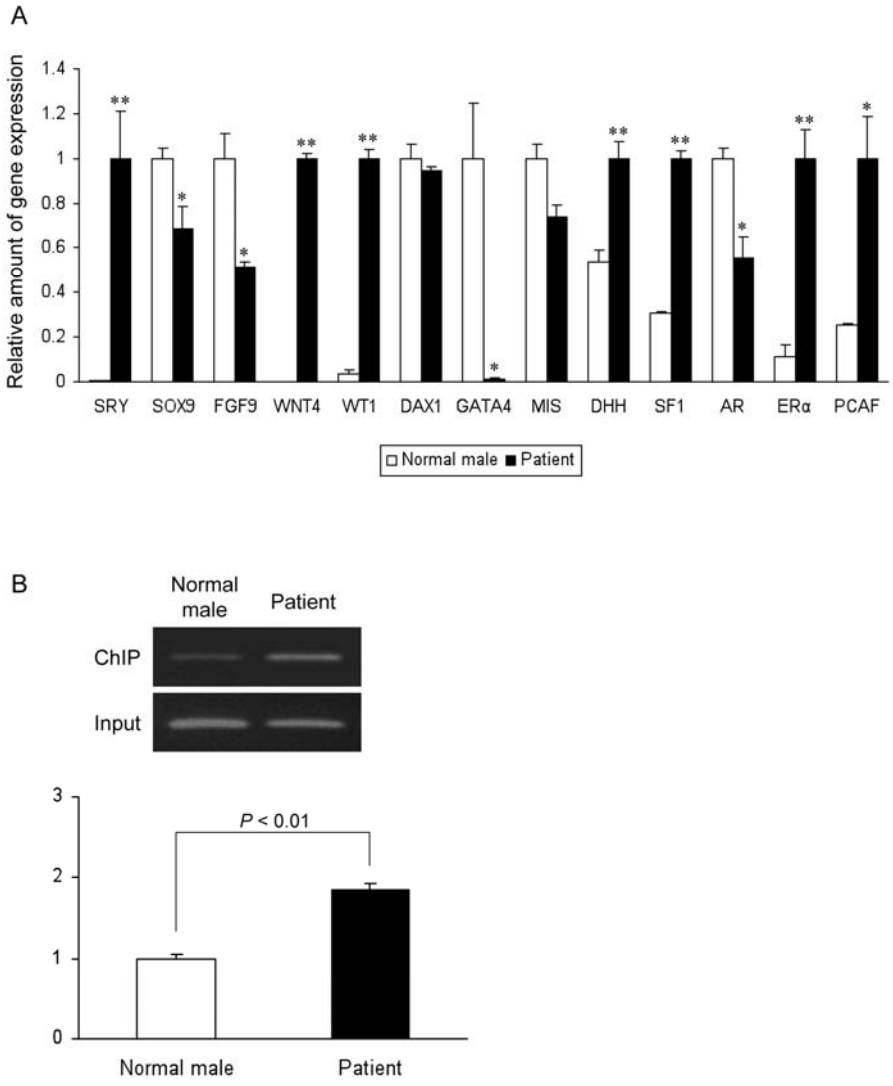

Fig. I-4

Quantitative RT-PCR analysis of the mRNA expressions of the sex-determining genes and *PCAF* in the fibroblasts. The data were analyzed using Student's *t*-test and represented as the mean ± SE ($n = 3$). **$p < 0.01$ and *$p < 0.05$ versus the normal male. (**B**) Acetylation status of histone H3 in *SRY* ($p = 0.0021$). The immunoprecipitated DNA was subjected to PCR. Input DNA, before immunoprecipitation, was used as the control. The data were analyzed using Student's *t*-test and represented as the mean ± SE ($n = 3$). $p < 0.01$ versus the normal male.

Table I-1 Primer design

Target gene	Primer	Product size (bp)	Annealing temperature (℃)	Number of PCR cycles
DNA analysis	(Nakahori *et al*. 1991)			
PABY				
Forward	5’-GTACTACCTTTAGAAAACTAGTATTTTCCC-3’	Y=947		
Reverse	5’-CTGCAGAAACAAGCTCATCAGCGTGACTAT-3’ 5’-GAATTCTTAACAGGACCCATTTAGGATTAA-3’	X=771		
SRY				
Forward	5’-CAGTGTGAAACGGGAGAAAACAGT-3’	270		
Reverse	5’-CTTCCGACGAGGTCGATACTTATA-3’			
AMGL				
Forward	5’-CTGATGGTTGGCCTCAAGCCTGTG-3’	Y=788		
Reverse	5’-TAAAGAGATTCATTAACTTGACTG-3’	X=977		
DYZ3				
Forward	5’-ATGATAGAAACGGAAATATG-3’	170		
Reverse	5’-AGTAGAATGCAAAGGGCTCC-3’		57	33
DYS139				
Forward	5’-ATTTCCTTGTCCAATTATCTTCT-3’	1650		
Reverse	5’-AGTTCCATCCTTTATATAACTGT-3’			
DYS132				
Forward	5’-TAGGTGGTGGTGTTGGCTGA-3’	420		
Reverse	5’-ACTCTGAGGTGCAGTTAGCC-3’			
DYS1				
Forward	5’-AATAGAGCCTTATCAGCAGC-3’	820		
Reverse	5’-AGTCAGTCTGGATGTTTCAG-3’			
DYZ1				
Forward	5’-AATTTGAGCATTCGTGTCCATTCT-3’	1024		
Reverse	5’-AATGCCCTTGAATTAAATGGACT-3’			
Sequencing	(Gimelli *et al*. 2006)			
SRY				
Forward	5’-GAATCTGGTAGAAGTGAGTTTTGGA-3’	780	65	30
Reverse	5’-TAAGAAAGTGAGGGCTGTAAGTTATCGT-3’			
ChIP assay				
SRY				
Forward	5’-TACAGGCCATGCACAGAGAG-3’	179	57	30
Reverse	5'-TCTTGAGTGTGTGGCTTTCG-3'			

Table I-2 Primers used in real-time RT-PCR

Target gene		Primer	Reference
SRY	Forward	5’-CAGTGTGAAACGGGAGAAAACAGT-3’	Nakahori *et al*. 1991
	Reverse	5’-CTTCCGACGAGGTCGATACTTATA-3’	
SOX9	Forward	5’-ATCTGAAGAAGGAGAGCGAG-3’	Kojima *et al*. 2008
	Reverse	5’-TCAGAAGTCTCCAGAGCTTG-3’	
FGF9	Forward	5’-GCAGCTATACTGCAGGACTG-3’	Knower *et al*. 2007
	Reverse	5’-CAGTTCAGGTACTTTGTCGG-3’	
WNT4	Forward	5’-GCTGGAACTGCTCCACACTCG-3’	Kojima *et al*. 2008
	Reverse	5’-CCCGCATGTGTGTCAGGATGG-3’	
WT1	Forward	5’-GGCATCTGAGACCAGTGAGAA-3’	Kojima *et al*. 2008
	Reverse	5’-GAGAGTCAGACTTGAAAGCAGT-3’	
DAX1	Forward	5’-CCGCTTGCAGTTCGAGACTGTG-3’	Kojima *et al*. 2008
	Reverse	5’-CTCATGGTGAACTGCACTACTG-3’	
GATA4	Forward	5’-CTGGCCTGTCATCTCACTACG-3’	Kojima *et al*. 2008
	Reverse	5’-GGTCCGTGCAGGAATTTGAGG-3’	
MIS	Forward	5’-CTATGAGCAGGCCTTCCTGG-3’	Kojima *et al*. 2008
	Reverse	5’-CCTCCAGGTGTAGGACCAC-3’	
DHH	Forward	5’-CTACTACGAGTCCCGCAACC-3’	Knower *et al*. 2007
	Reverse	5’-CGCCAAAACCCAGTCTCC-3’	
SF1	Forward	5’-ACGCTGCTGCAGAACTGCTGG-3’	Kojima *et al*. 2008
	Reverse	5’-TTGCGGGGCATCTCGTTGCCC-3’	
AR	Forward	5’-GGTGAGCAGAGTGCCCTATC-3’	–
	Reverse	5’-GAAGACCTTGCAGCTTCCAC-3’	
ERα	Forward	5’-CAGGTGCCCTACTACCTGGA-3’	–
	Reverse	5’-TCCTTGGCAGATTCCATAGC-3’	
PCAF	Forward	5’-AGAACATTGCTTCGCTCGG-3’	Kurebayashi *et al*. 2000
	Reverse	5’-TGCCTCAAGTCCAGAAGAGG-3’	
GAPDH	Forward	5’-CATGGAGAAGGCTGGGGCTC-3’	Truong *et al*. 2006
	Reverse	5’-CACTGACACGTTGGCAGTGG-3’	

–: I designed specific primers using Primer3.

Table I-3 Summary of DNA analysis

Locus	Normal male	Patient		
		Blood	Skin	Gonad
PABY	+	+	+	+
SRY	+	+	+	+
AMGL	+	+	+	+
DYZ3	+	+	+	+
DYS139	+	+	+	+
DYS132	+	+	+	+
DYS1	+	+	+	+
DYZ1	+	+	+	+

+: present

Eight different loci (*PABY*, *SRY*, *AMGL*, *DYZ3*, *DYS139*, *DYS132*, *DYS1*, and *DYZ1*) of the Y chromosome encompassing the distal short arm to the distal long arm were all present. Y chromosome deletion was not noted in samples from peripheral blood, skin, and gonadal tissue.

Chapter II:

Global gene profiling and comprehensive bioinformatics analysis of a 46,XY female with pericentric inversion of the Y chromosome

INTRODUCTION

Disorders of human sexual determination/differentiation trigger malformations of the internal and/or external genitalia ranging from genital ambiguity to complete sex reversals such as those in the XY female and the XX male. DSD are not rare; they occur in approximately 0.2–0.3% of the population [Fujimoto and Hayashi, 1983]. The pathophysiology of DSD is complex, including numerical/structural sex chromosome aberrations, sex differentiation-related gene mutations, disorders of steroid formation/action, defects of androgen receptors, and other factors, and many unexplained phenomena have been encountered in clinical experience [Hoshi *et al*., 1998a, 1998b].

XY females are rare individuals who carry a Y chromosome but are phenotypically female. The occurrence of XY gonadal dysgenesis, first described in 1955 by Swyer, is estimated at 1:20,000 [Robinson and Linden 1993]. Individuals with this condition appear to be normal females at birth, but they do not develop secondary sexual characteristics at puberty, do not menstruate, and have streak gonads.

SRY, a testis-determining gene [Sinclair *et al*., 1990; Koopman *et al*., 1991], is located on the short arm of the Y chromosome and plays a critical role in initiating testis formation during gonadal development [Koopman *et al*., 1990, 1991]. However, the mode of *SRY* action is poorly understood, even though 20 years have passed since the *SRY* gene was identified in 1990, and the presence of missense mutation [Hawkins *et al*., 1992a; Zeng *et al*., 1993; Imai *et al*., 1999; Okuhara *et al*., 2000; Shahid *et al*., 2004], nonsense mutation [Hawkins *et al*., 1992a; Müller *et al*., 1992; Iida *et al*., 1994; Giuffrè *et al*., 2004], or frame shift mutation [Jäger *et al*., 1990; Kellermayer *et al*., 2005] in the *SRY* gene has been detected in only about 10–20% of XY females [Hawkins *et al*., 1992b; Lim *et al*., 1998]. Therefore, the cause of DSD has been highly controversial.

Studying an XY female with no detectable mutations in the *SRY* gene may be relevant to understanding the alterations in genes downstream of *SRY* and/or in X chromosomal or autosomal genes in the sex-determining pathway. To that end, it is necessary to clarify any differences in gene expression between XY females and normal males for the entire genome, and not only those genes related to sex differentiation.

To tackle this problem, I compared gene expression patterns in cultured fibroblasts from a normal adult male to those from the patient, an XY female with a pericentric inversion of the Y chromosome but without functional mutation in *SRY*, using Affymetrix GeneChip technology representing 22,283 distinct human genes. Microarray-based gene expression profiling enables us to simultaneously profile the expression of all genes expressed in an individual organism. Given the complexity of DSD and our limited understanding of its pathogenetic mechanisms, I considered that an exhaustive analysis of the changes occurring in the gene expression profile between a normal adult male and a DSD patient would be very useful. In itself, however, microarray analysis of the alterations in gene expression is not sufficient. It is also necessary to translate the information on individual genes into a broader characterization using systematic functional annotation. For example, GO analysis [Ashburner *et al*., 2000] including three independent ontologies—the Biological Process, Molecular Function and Cellular Component ontologies—enables us to annotate altered genes biologically, while GenMAPP [Dahlquist *et al*., 2002] is a new tool for viewing and analyzing microarray data on biological pathways. It is important to analyze the changes in individual gene expressions from this collective, global perspective, and I therefore considered that GO-based or pathway-based mapping of the gene expression changes in a DSD patient could provide an understanding of the molecular-level differences between normal adult males and individuals with DSD.

In the present study, using a comprehensive bioinformatics analysis, I attempted to

reveal the molecular-level changes that contribute to DSD. Global genes profiling analysis is very important in the analysis of several birth defects including defects in sexual differentiation, and the findings of my study provide some insight into the mechanisms leading to DSD.

MATERIALS AND METHODS

Written informed consent was obtained from the patient and her parents, and the study was approved by the institutional ethics board of Hokkaido University Graduate School of Medicine.

Subject

The patient, a 17-year-old Japanese woman (gravida 0, para 0) of normal height, was born to healthy parents as a low-birth-weight infant (1,910 g) with normal female external genitalia at 39 weeks of gestation. She was referred to our institution for primary amenorrhea with neither pubic nor axillary hair and underdeveloped breasts. Pelvic MRI disclosed a uterus of normal size and shape which appeared to be functional based on withdrawal bleeding in response to administration of hormonal agents. Ovaries were not identified on MRI scans, and there were no signs of a mass on either side of the inguinal region. She had elevated gonadotropin levels (LH = 117.1 mIU/mL, FSH = 417.5 mIU/mL) and extremely low levels of steroid hormones (testosterone = 0.22 ng/mL, progesterone = 0.35 ng/mL, estradiol (E2) = undetectable). Her past history was notable for membranous nephropathy and an inguinal hernia requiring surgical repair at age two. Detailed cytogenetic analysis revealed that she had the 46,X, inv(Y)(p11.2q11.2) ish inv(Y)(SRY+,DYZ3+,DYZ1+) karyotype and a whole Y chromosome. The sequence of the open reading frame of the *SRY* gene had a synonymous SNP (c.465C>T; p.S155S), but no other mutation associated with a functional abnormality was detected. Histologic examination of gonadal tissues yielded a diagnosis of streak gonad. There were SRY-positive cells in a mostly fibrous stroma.

Cell Culture

Primary cultures were established from the elbow skin and gonadal tissue. A human male cell line from skin fibroblasts originating from a normal young man was purchased from Cell Systems (Kirkland) and used as a normal male control. Cells were cultured in α-MEM (Invitrogen-Gibco) supplemented with 10% FBS (Biowest) and incubated in 25 cm^2 cell culture flasks at 37ºC in a humidified atmosphere with 5% CO_2. The medium was changed regularly, and cells were grown to confluence to standardize the state of proliferation as much as possible.

RNA Extraction

Total cellular RNA was extracted from cultured skin and gonadal fibroblast cells using an AllPrep™DNA/RNA Mini kit (Qiagen). Then, RNA samples were treated with RNase-free DNase to eliminate genomic DNA contamination.

Gene Expression Profiling

For gene expression analysis of skin and gonadal fibroblasts, I used a GeneChip® system with a Human Expression Array U133A spotted with 22,283 probe sets (Affymetrix). The samples were prepared for the array hybridization as follows. Biotin-labeled cRNA probes were generated from 5 μg of DNase-treated total RNA. After fragmentation, the biotinylated cRNA probe was hybridized to HG-U133A arrays at 45°C for 16 hr. The arrays were washed, stained with streptavidin-phycoerythrin, and scanned with a probe array scanner. The scanned chip was analyzed using GeneChip Analysis Suite software (Affymetrix). A representative signal value and detection *p* value for each probe set were calculated with the Microarray Suite version 5 (MAS5; Affymetrix) algorithm [Tabuchi *et al*., 2006].

Data were imported into GeneSpring software version GX7.3.1 (Agilent), and the

following normalizations were applied: data transformation setting measurements less than 0.01 to 0.01 and per-chip normalization (total chip normalization to the 50th percentile). The GeneChip control probes designed by Affymetrix, which are denoted by IDs containing AFFX-, were eliminated from all probes. The flags (present, marginal, or absent) were assigned by Affymetrix treatment of the arrays. A value of $p > 0.06$ indicates that the gene is not detected at a level significantly different from background and is thus designated as *absent*; a value of p between 0.06 and 0.04 results in a designation of *marginal*; and a value of $p < 0.04$ indicates that a gene is *present*. Only signals designated present or marginal in at least three of four chips were used in further analysis. This procedure facilitated the elimination of transcripts with very low signals in both treatments (these transcripts were designated absent).

I extracted those genes whose expression levels differed between the patient and a normal male (n = 2). A Welch's *t*-test used to compare the expression levels between the patient and the normal male, and p values less than 0.05 were regarded as significant.

Gene annotation for Affymetrix platforms (U133A) was obtained from the Affymetrix – NetAffyx Analysis Center (http://www.affymetrix.com/analysis/index.affx), NCBI Entrez Gene (http://www.ncbi.nlm.nih.gov/sites/entrez?db=gene), GO (http://www.geneontology.org/index.shtml), GenMAPP pathway (http://www.genmapp.org/) and TRED (Transcriptional Regulatory Element Database; http://rulai.cshl.edu/cgi-bin/TRED/tred.cgi?process=searchTFGeneForm).

To identify significantly over-represented GO categories in differentially expressed genes, Fisher's exact test was performed using the GeneSpring GO browser tool, which iterates over all GO terms. *P* values less than 0.05 were regarded as significant. The resulting statistically significant GO terms included in the Biological Process ontology were grouped according to the GO classification defined in the L2L Microarray

Analysis Tool (http://depts.washington.edu/l2l/about.html).

To examine the signaling network in differentially expressed genes, I utilized a list of the genes and their functions, cellular locations and signaling relations in the human signaling network as reported by Cui *et al.* [2007], and the network visualization was done by using the Cytoscape program (http://www.cytoscape.org/) [Shannon *et al*., 2003]. The cerebral (Cell Region-Based Rendering And Layout) plug-in was used for the layout with subcellular localization annotation (http://www.pathogenomics.ca/cerebral/) [Barsky *et al*., 2007].

In order to detect significant differences at the level of functional categories, such as pathway genes and transcriptionally regulated genes with a cis-regulatory element in the promoter, I performed Parametric Analysis of Gene Set Enrichment (PAGE) as reported by Kim and Volsky [2005]. This test was implemented by calculating a Z score for each gene set, which measures the deviation of the average log-ratio for genes in the category from the genome-wide average, in units of the standard deviation. The Z score for each category was calculated as follows:

$$Z = (\bar{x} - \mu) / (\sigma / \sqrt{n}) \ ,$$

where μ and σ represent the mean and the standard deviation of the total fold change values of quality controlled genes in a given microarray data set, respectively, $\bar{x}$ is the mean of the fold change values of genes for a given gene set and n is the size of a given gene set. The predefined gene sets were created from GenMAPP and TRED. I used Excel2003 (Microsoft, Redmond, WA, USA) to obtain the Z score and the corresponding *p* value.

RESULTS

Gene Expression Profile by Microarray Technology

Exhaustive analysis using DNA microarrays representing 22,283 different probe sets showed striking differences in the gene expression profiles between a normal male and the patient. When only those genes with significantly different expression levels ($p < 0.05$) were selected, a total of 1,820 probe sets were identified as showing significantly differential expression between the skin fibroblasts of the patient and the normal male. Of these 1,820 transcripts, 988 were upregulated and 832 were downregulated in the patient vs. the normal male.

Feature Extraction of Altered Genes by GO analysis

The genes differentially expressed between the patient and the normal male were subjected to functional clustering according to the GO classification. I first grouped the genes belonging to the GO Biological Process category into 18 major categories according to the L2L Microarray Analysis Tool. This pie chart was able to represent at a glance the entire spectrum of cellular functions associated with the Biological Process ontology in the patient, and thus there was not much difference in feature extraction by means of GO classification defined in the L2L Microarray Analysis Tool between the upregulated genes and the downregulated genes in the patient (Fig. II-1A, B).

Next I viewed each of the GO categories separately (Biological Process, Molecular Function, and Cellular Component). The results for the Biological Process category are shown in Tables II-1 and II-2. The GO terms were ranked by *p* value, and only the top 20 GO terms from the lists of both the significantly upregulated and downregulated genes were included in Table II-1. It is striking that GO terms categorized in the development category of the Biological Process ontology were included in the list of

upregulated genes (shown in bold) but not included in the list of downregulated genes. Table II-2 represents only those GO terms that were included in the development category and whose *p* values were less than 0.01. The sex determination was included (shown in bold). The results for the Molecular Function ontology are shown in Table II-3. Only GO terms whose *p* values were less than 0.001 in the significance lists for both upregulated and downregulated genes were included in Table II-3. GO terms of the transcriptional repressor activity, transcription corepressor activity, transcription cofactor activity and transcription factor binding were included in the list of upregulated genes (shown in bold). The results for the Cellular Component ontology are shown in Table II-4. Only GO terms whose *p* values were less than 0.001 in the significance lists for both upregulated and downregulated genes were included in Table II-4. A GO term of the MHC class I protein complex was included in the list of upregulated genes (shown in bold) and GO terms of the condensed chromosome, pericentric region and the chromosome, pericentric region were included in the list of downregulated genes (shown in bold).

Altered Gene Network

The genes differentially expressed between the patient and the normal male were used to construct a gene network by referring to the data reported by Cui *et al.* [2007]. Because the up- and downregulated proteins interacted with each other, I included both up- and downregulated genes in a single gene network. This network included 85 proteins connected by 151 interactions (Fig. II-2). The nodes of KIT, KITLG, GRB10, ID1, ID2, ID3, ID4, HES1, DAB2 and TGFBR1 were evident in this network. These genes were connected to each other and a series of genes was shown to be upregulated.

Pathway Changes

Normalized genes were grouped into functional categories using PAGE and the pathway data from GenMAPP, forming 79 functional pathways. The up- or downregulation of these functional pathways in the patient (compared to the normal male) was targeted and *p* values less than 0.05 were regarded as significant. The patient showed 25 altered pathways, 13 of which were significantly decreased, and 12 of which were significantly increased (Fig. II-3). The transforming growth factor-β (TGF-β) signaling pathway was increased, while the electron transport chain pathway and Wnt signaling pathway were decreased.

Significant Changes of Transcription Factors

In order to determine whether or not each transcription factor was active in the patient, I utilized PAGE and the data from TRED. 63 transcription factors were extracted and filtered to include only those with *p* values less than 0.05, which were regarded as statistically significant. As a result, 32 transcription factors (12 decreased and 20 increased) were found to be significantly different between the patient and the normal male (Fig. II-4). Interestingly, ESR1 and WT1, which are associated with sex development, were included.

DISCUSSION

It has been reported that the prevalence of males with pericentric inversions of the Y chromosome is approximately 1:1,000 in the general population [Zeuthen and Nielsen, 1973; Tóth *et al*., 1984], and pericentric inversion of the human Y chromosome is considered a structural variant that does not influence phenotype [Schmid 1985; Krishna Murthy *et al*., 1989]. Therefore, I considered that the patient could have had the same XY cells as normal males at the cellular level. If so, despite the XY cells being identical in origin, what changes could have occurred at the molecular level between a normal male and an XY female?

In the present study, using comprehensive bioinformatics analyses, I tried to provide a molecular-level comparison between a normal XY male and an XY female. This study was performed using human skin fibroblasts derived from an XY female with a functional *SRY* and pericentric inversion of the Y chromosome, and the results were compared with those for a normal male. Skin fibroblasts were used for the following reasons: i) it is difficult to obtain normal male gonads as a control; ii) chromosome examinations indicated that the skin and gonadal fibroblasts of this patient had an identical karyotype; and iii) *SRY* was expressed in both skin and gonadal fibroblasts of this patient (data not shown). My objective was to further clarify the overall changes in gene expression and to provide clues for exploring the complex pathogenesis of XY females without *SRY* functional mutation. For that purpose, several methods of analysis were used to examine different aspects of gene expression.

Exhaustive analysis using DNA microarrays revealed striking differences in the gene expression profiles between a normal male and the patient. Of these 22,283 probe sets, I identified 1,820 probe sets (988 upregulated and 832 downregulated) that showed significant differences in transcription levels between the patient and the normal male.

Interestingly, not only genes on the Y chromosome in which pericentric inversion occurred, but also numerous genes on autosomal or X chromosomes were altered in the patient. In order to untangle the complex changes and extensive interaction among the differentially expressed genes, in-depth analyses using a data mining technique were required. Here I describe results with a focus on genes associated with not only sex determination/differentiation but also development and morphogenesis, and genes which were localized near the inversion breakpoints of chromosome Y.

Using a gene network to study the functional relationship between related genes offers novel perspectives on the molecular basis of the phenomenon in question. The genes differentially expressed between the patient and the normal male were used to generate a gene network by referring to the data of Cui *et al*. [2007]. The nodes of KIT, KITLG, GRB10, ID1, ID2, ID3, ID4, HES1, DAB2 and TGFBR1 were connected to each other and the increased expression of a series of genes was revealed. Signaling from the KIT receptor tyrosine kinase is essential for primordial germ cell growth both *in vivo* and *in vitro*, and most cell-cell interactions during embryonic development involve the receptor tyrosine kinase and TGF-β pathways [Pires-daSilva and Sommer, 2003]. The membrane-bound protein encoded by TGFBR1 binds TGF-β and forms a heterodimeric complex with the TGF-β II receptor [Franzén *et al*., 1993; Johnson *et al*., 1995] for activation of their kinase (signaling) function. Pathways which were involved in primordial germ cell growth and/or embryo development were altered.

The GO Consortium [Ashburner *et al*., 2000] includes three independent ontologies that respectively describe biological processes, molecular functions and cellular components. The goal of the GO Consortium is to produce a dynamic, controlled vocabulary that can be applied to all eukaryotes even as our knowledge of the roles of cellular genes and proteins continues to accumulate and change. Using the GO classification system, I tried to extract the features of the altered genes in this patient.

As a result, I determined that numerous genes in the development category of the Biological Process ontology were upregulated. Notably, these upregulated genes included several genes for sex determination. It was significant for this patient, and for DSD patients generally, that the GO term of sex determination was extracted as one of the features of the altered genes. In other words, the precise GO term which would be expected to represent the cause of DSD was altered. In the Molecular Function ontology, genes associated with transcriptional repressor activity, transcription corepressor activity, transcription cofactor activity and transcription factor binding, which were transcription-associated GO terms, were upregulated, and these changes could play a role in the expression of a huge number of genes either directly or indirectly. In the Cellular Component ontology, genes related to the MHC class I protein complex were upregulated. It has been reported that engagement of CD99 induces upregulation of an MHC class I molecule on the surface of human thymocytes [Choi *et al*., 1998]. The *CD99* gene was expressed at a 2.198-fold higher level in this patient than in the normal male control (p = 0.0179). Therefore, the upregulation of the MHC class I protein complex may be attributed to the elevated levels of *CD99* expression. In addition, *CD99* is located on the distal part of Yp, namely, Ypter-Yp11.2 [Buckle *et al*., 1985], which means that *CD99* was located adjacent to the chromosomal position at which the inversion occurred [inv(Y)(p11.2q11.2)]. The pericentric inversion of the Y chromosome may affect the expression of *CD99*. Upregulation of the MHC class I protein complex induced by upregulation of *CD99* may be the event which came under the direct influence of the pericentric inversion of the Y chromosome. Meanwhile, the genes for condensed chromosome, pericentric region and chromosome, pericentric region were downregulated. Surprisingly, however, neither of these sets of genes included the genes on the Y chromosome. Instead, they included the genes on chromosomes 1, 2, 4, 10, 15, 17, and X (data not shown). It was interesting that

pericentric inversion of the Y chromosome may affect the pericentric regions of other chromosomes.

PAGE [Kim and Volsky, 2005] is a useful microarray analysis method. It is statistically sensitive and requires less computation than many other programs. It can identify biological themes that are significantly changed from a microarray data set irrespective of the microarray data analysis methods or microarray platforms used, and it is useful for comparing multiple microarray data sets. Meanwhile, GenMAPP [Dahlquist *et al*., 2002] is a new tool for viewing and analyzing microarray data on biological pathways. It displays gene expression data on pathways by color-coding the genes based on data and criteria provided by the investigator, and also has graphics tools for constructing and modifying pathways. In addition, it provides access to annotation for genes and a connection with pathway experts. In this study, normalized genes were grouped into functional categories using PAGE and the pathway data from GenMAPP, forming 25 (13 decreased and 12 increased) altered functional pathways in the patient (compared to a normal male). The functional gene pathway analysis (PAGE) revealed that not only at the gene level, but also at the pathway level, there were clear differences between the patient and the normal male. For example, the electron transport chain pathway was significantly decreased. This pathway includes the *ANT3Y* gene. The ADP/ATP translocase gene (*ANT3*) is located on the pseudoautosomal region of the X (Xp22.32) and Y (Yp11.3) chromosomes, escapes X inactivation, and is transcribed from the Y chromosome and from both the active and the inactive X chromosome [Slim *et al*., 1993]. This was the alteration of the pathway involving the Y chromosome with pericentric inversion, and this alteration may have been affected by the pericentric inversion. At the same time, it is interesting that the TGF-β signaling pathway and Wnt signaling pathway were altered, because it has been reported that only a few signaling pathways are required to generate the bewildering number of cell types

and patterns, and most cell-cell interactions during embryonic development involve the Hedgehog, Wnt, TGF-β, receptor tyrosine kinase, Notch, JAK/STAT and nuclear hormone pathways [Pires-daSilva and Sommer, 2003].

In order to understand gene regulation, accurate and comprehensive knowledge of transcriptional regulatory elements is essential. TRED can provide good training datasets for further genome-wide cis-regulatory element prediction, assist in detailed functional studies, and facilitate the deciphering of gene regulatory networks. In order to determine whether or not each transcription factor was active in this patient, I utilized PAGE and the data from TRED. Analysis of the gene sets regulated by transcription factors (PAGE) revealed that 32 transcription factors (12 decreased and 20 increased) were significantly different between the patient and the normal male. The expression levels of all of these transcription factors were altered, a level of change which would have an incalculable impact on gene expression in any organism. Interestingly, ESR1 [Corbo *et al*., 2007] and WT1 [Klattig *et al*., 2007], which were associated with sex development, were also included among the altered transcription factors.

I observed consistent and characteristic differences in the gene expression patterns between the DSD patient and a normal male control. Only the most important conclusions taken from these data were discussed, in brief, above. It was of special interest to me that alterations of genes associated with development, including sex determination and organ morphogenesis genes, were observed in the patient and that alterations of the functional pathways in which most cell-cell interactions during embryonic development are involved were observed in the patient. The alterations of gene expression may have been related in some way to the inversion of the Y chromosome, because the present patient had only inversion but no deletion of the Y chromosome; that is, the gene dosage was unchanged. Several aspects of my analyses may be widely extensible, and gene-profile comparisons between a patient and a normal

male control provide an interesting way to explore the complex pathophysiology of XY females.

In conclusion, alterations in the expression of numerous genes at the developmental stage—including alterations at both the gene and pathway levels—may persist as a vestige of anomalies of sex differentiation that presumably began in the fetal period. This study indicates that a data mining technique using bioinformatics contributes to identification of not only genes responsible for birth defects but also DSD-specific pathways, and that this kind of analysis is an important tool for clarifying the pathophysiology of human idiopathic XY gonadal dysgenesis. These findings could serve as one of the basic data set which will be used for future investigations, because the accumulation of differences in gene expression between patients and normal control leads to understanding of the pathogenic mechanism of DSD. My approach suggests a number of important avenues for future follow-up research.

SUMMARY

XY females are rare individuals who carry a Y chromosome but are phenotypically female. In approximately 80–90% of these cases, there are no mutations in the *SRY* gene, a testis-determining gene on the short arm of the Y chromosome, and the pathophysiology of XY females without *SRY* mutation remains unclear. In this study, I used a molecular data mining technique to analyze the pathophysiology of an XY female with functional *SRY* and pericentric inversion of the Y chromosome, and compared the results with those for a normal male. Interestingly, upregulations of numerous genes included in the development category of the Biological Process ontology, including genes associated with sex determination and organ morphogenesis, were seen in this patient. Additionally, the TGF-β signaling pathway and Wnt signaling pathway, in which most cell-cell interactions during embryonic development are involved, were altered. In conclusion, alterations in the expression of numerous genes at the developmental stage—including alterations at both the gene and pathway levels—may persist as a vestige of anomalies of sex differentiation that presumably began in the fetal period. This study indicates that a data mining technique using bioinformatics contributes to identification of not only genes responsible for birth defects but also DSD-specific pathways, and that this kind of analysis is an important tool for clarifying the pathophysiology of human idiopathic XY gonadal dysgenesis. My findings could serve as one of the basic data set which will be used for future follow-up investigations.

FIGURES AND TABLES

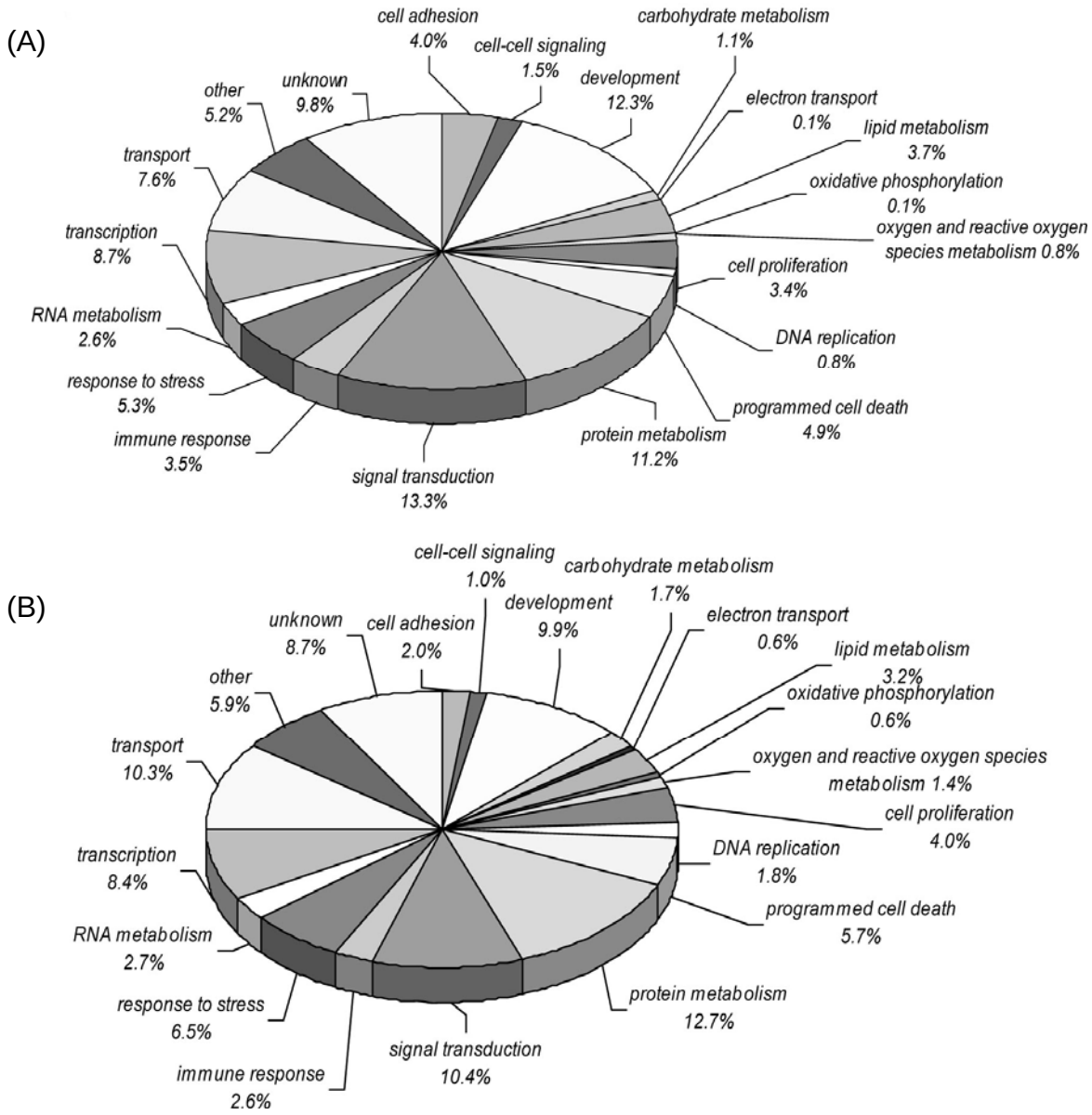

Fig. II-1

The proportion of the genes associated with the major GO terms which are included in the Biological Process ontology.

The pie charts show that the expression of some genes was significantly increased (A) and that of others was significantly decreased (B) in the patient ($p < 0.05$).

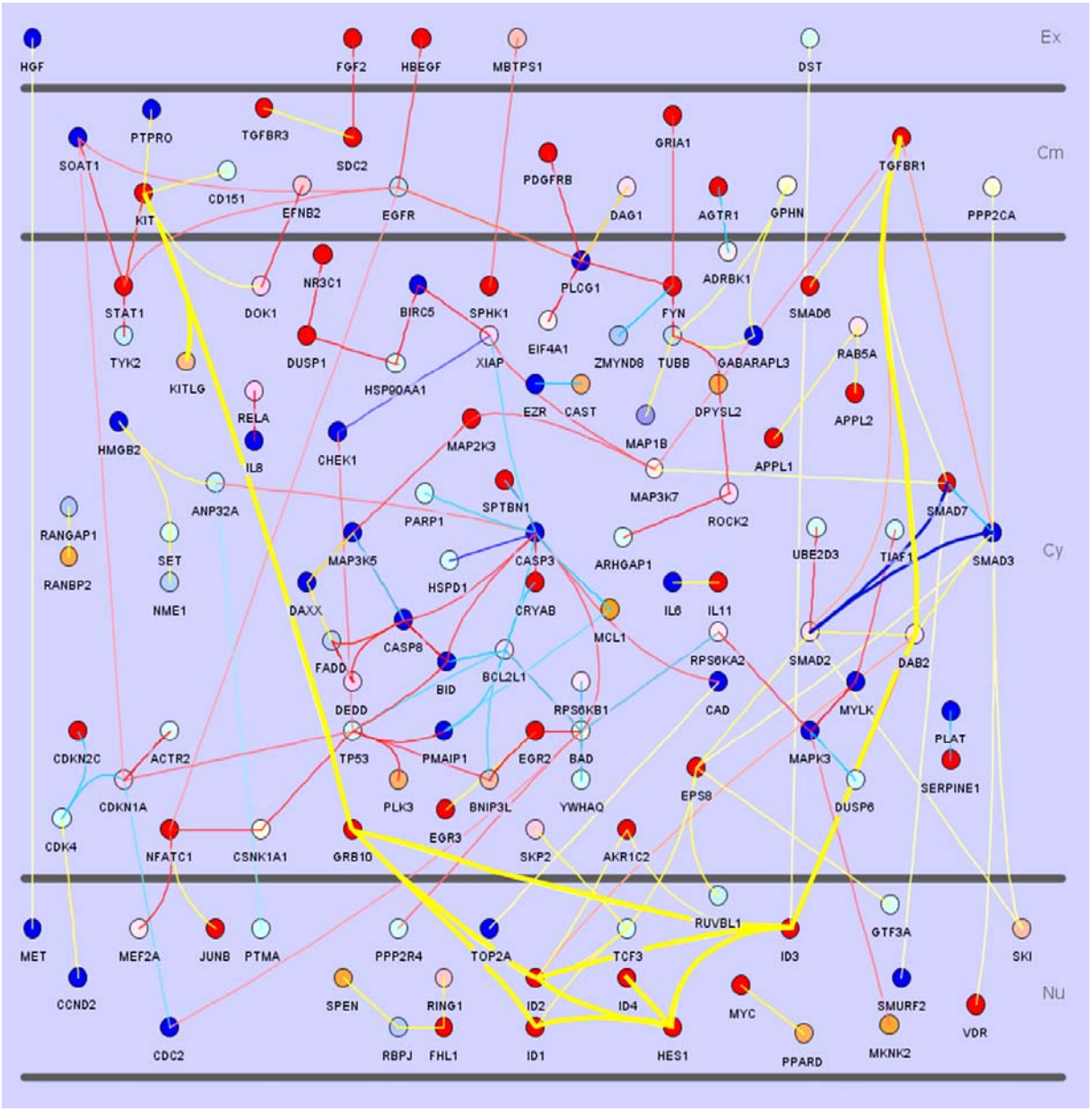

Fig. II-2

Gene network of 85 proteins from the up- and downregulated genes in the patient.

This network is connected by 151 interactions. The nodes of KIT, KITLG, GRB10, ID1, ID2, ID3, ID4, HES1, DAB2 and TGFBR1 were evident in this network (shown in bold). These genes were connected to each other, revealing that a series of genes was upregulated. FC indicates Fold Change.

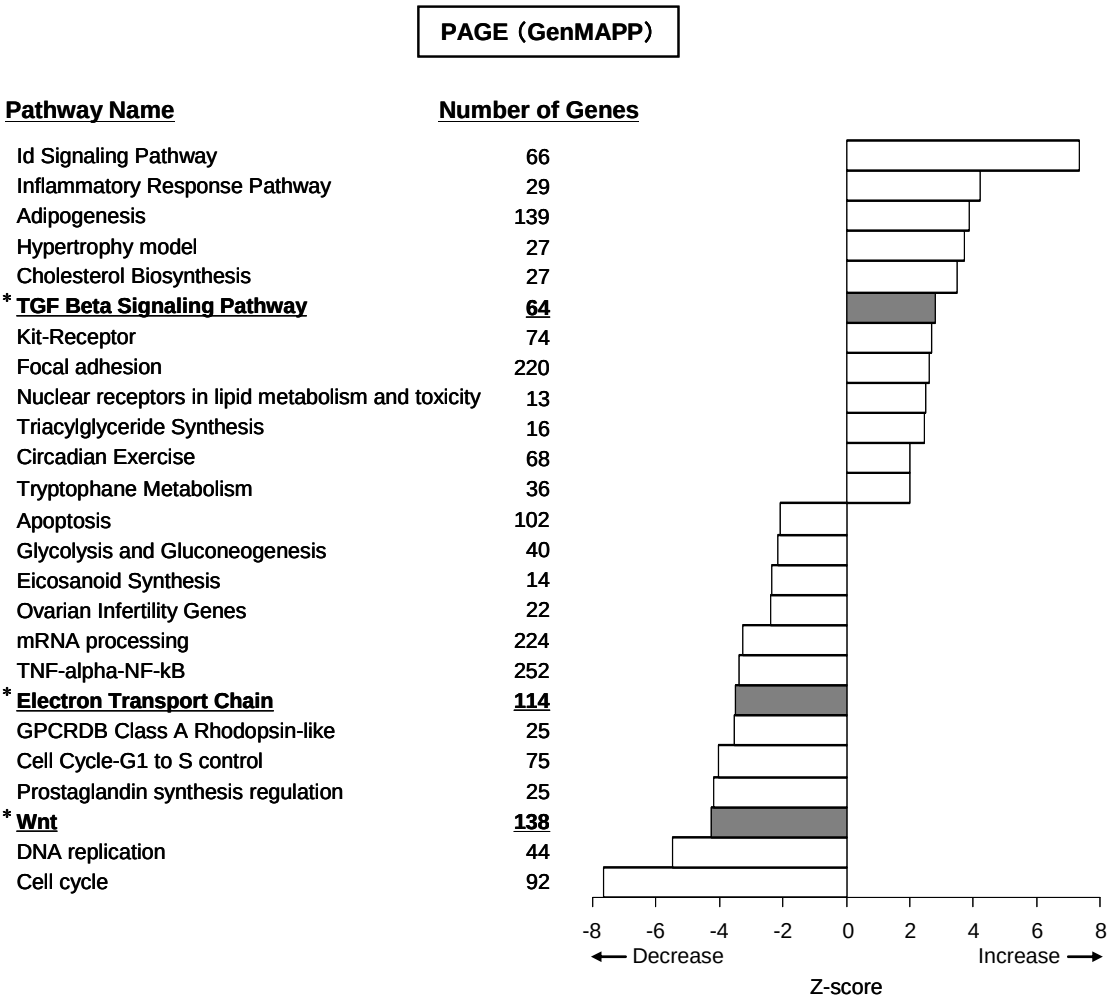

Fig. II-3

Significant changes of pathways between a normal male and the patient.

Significantly altered genes in the normal male and the patient were clustered into functional pathways. In the patient, there were 25 significantly altered pathways, of which 12 pathways were significantly upregulated and 13 pathways were significantly downregulated, compared to pathways in the normal male. Number of genes represents the number of altered genes included in each pathway. The pathways which were picked up in this study (*) are highlighted.

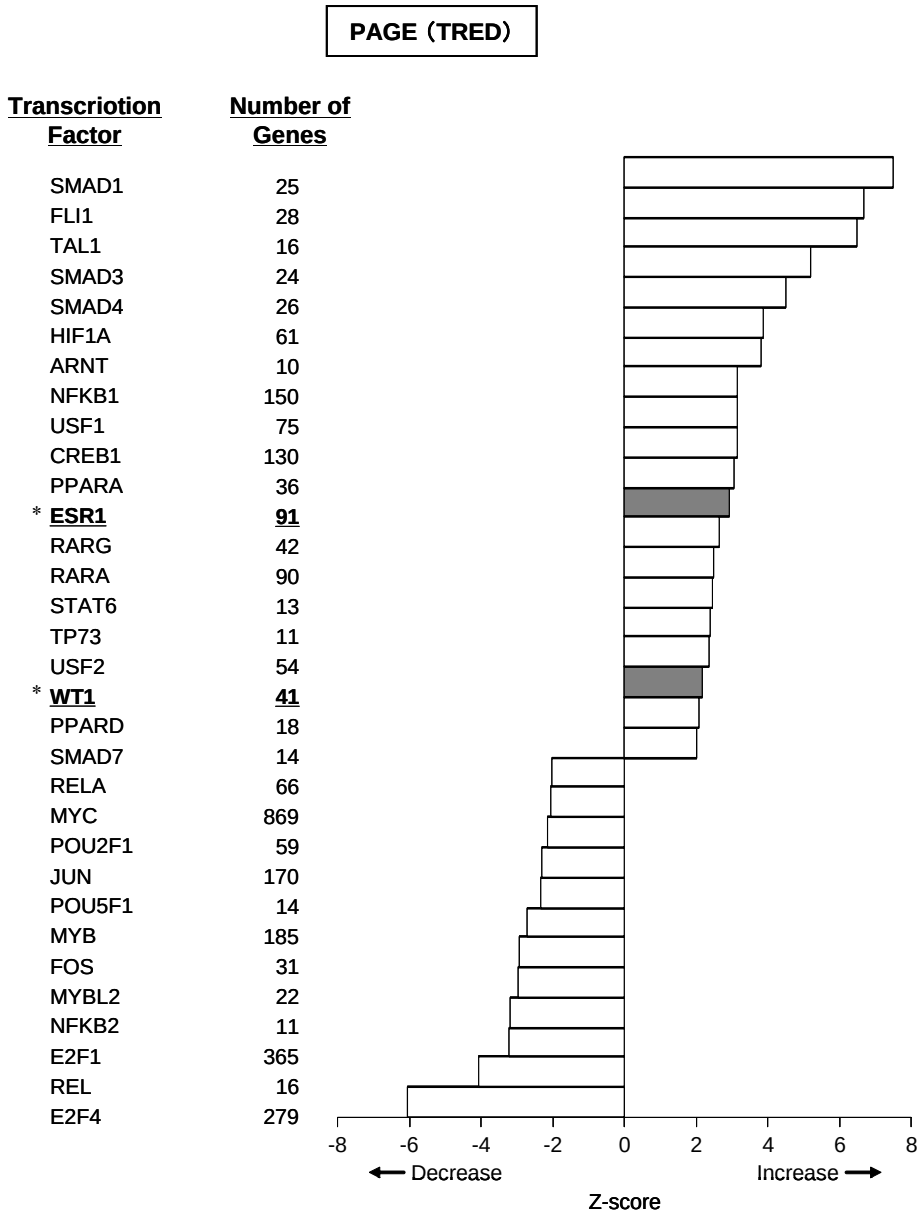

Fig. II-4

Significant changes of transcription factors between a normal male and the patient. Significantly altered genes between the normal male and the patient were classified under functional transcription factors. 32 transcription factors were significantly different between the patient and the normal male. 20 transcription factors were significantly upregulated and 12 transcription factors were significantly downregulated. Number of genes represents the number of altered genes regulated by each transcription factor. The transcription factors which were picked up in this study (*) are highlighted.

Table II-1 Top 20 Gene Ontology Biological Process terms statistically significantly altered in the patient

GO ID	GO Term	Genes in Category	Genes in List in Category	*p*-value
Upregulated in the patient				
GO:0050852	T cell receptor signaling pathway	30	12	5.31×10^{-9}
GO:0008354	Germ cell migration	13	8	2.81×10^{-8}
GO:0050858	Negative regulation of antigen receptor mediated signaling pathway	5	5	0.000000251
GO:0050860	Negative regulation of T cell receptor signaling pathway	5	5	0.000000251
GO:0050851	Antigen receptor-mediated signaling pathway	42	12	0.000000399
GO:0009887	**Organ morphogenesis**	747	62	0.0000174
GO:0050856	Regulation of T cell receptor signaling pathway	9	5	0.0000269
GO:0006817	Phosphate transport	115	17	0.0000334
GO:0007179	Transforming growth factor beta receptor signaling pathway	83	14	0.0000369
GO:0030334	Regulation of cell migration	128	18	0.0000393
GO:0001568	**Blood vessel development**	303	31	0.0000602
GO:0007178	Transmembrane receptor protein serine/threonine kinase signaling pathway	133	18	0.0000658
GO:0030335	Positive regulation of cell migration	39	9	0.0000742
GO:0048513	**Organ development**	1472	102	0.0000883
GO:0001944	**Vasculature development**	311	31	0.0000979
GO:0042675	**Cone cell differentiation**	3	3	0.00011
GO:0048712	**Negative regulation of astrocyte differentiation**	3	3	0.00011
GO:0051281	Positive regulation of release of sequestered calcium ion into cytosol	3	3	0.00011
GO:0046549	**Retinal cone cell development**	3	3	0.00011
GO:0042670	**Retinal cone cell differentiation**	3	3	0.00011
Downregulated in the patient				
GO:0050875	Cellular physiological process	13554	611	0.000000831
GO:0006979	Response to oxidative stress	130	19	0.00000175
GO:0012501	Programmed cell death	1219	82	0.00000903
GO:0008219	Cell death	1301	86	0.0000105
GO:0000278	Mitotic cell cycle	565	46	0.0000106
GO:0009117	Nucleotide metabolism	259	27	0.0000109
GO:0051641	Cellular localization	984	69	0.0000129
GO:0045210	FasL biosynthesis	5	4	0.0000143

(Continued on next page)

Table II-1 (Continued)

GO ID	GO Term	Genes in Category	Genes in List in Category	*p*-value
GO:0042752	Regulation of circadian rhythm	5	4	0.0000143
GO:0016265	Death	1313	86	0.0000148
GO:0051649	Establishment of cellular localization	959	67	0.0000195
GO:0045454	Cell redox homeostasis	69	12	0.000024
GO:0006800	Oxygen and reactive oxygen species metabolism	169	20	0.0000245
GO:0006915	Apoptosis	1208	79	0.0000358
GO:0006259	DNA metabolism	1061	71	0.0000445
GO:0006790	Sulfur metabolism	100	14	0.0000645
GO:0008104	Protein localization	1094	71	0.000115
GO:0008632	Apoptotic program	176	19	0.000135
GO:0044248	Cellular catabolism	870	59	0.00014
GO:0000226	Microtubule cytoskeleton organization and biogenesis	111	14	0.000202

'Genes in Category' represents number of genes included in the GO category; 'Genes in List in Category' represents number of altered genes in the GO category. GO terms are ranked by *p* value. GO terms categorized into development are shown in bold.

Table II-2 Statistically significantly upregulated Gene Ontology terms categorized into development (Biological Process) in the patient ($p < 0.01$)

GO ID	GO Term	Genes in Category	Genes in List in Category	*p*-value
GO:0009887	Organ morphogenesis	747	62	0.0000174
GO:0001568	Blood vessel development	303	31	0.0000602
GO:0048513	Organ development	1472	102	0.0000883
GO:0001944	Vasculature development	311	31	0.0000979
GO:0042675	Cone cell differentiation	3	3	0.00011
GO:0048712	Negative regulation of astrocyte differentiation	3	3	0.00011
GO:0046549	Retinal cone cell development	3	3	0.00011
GO:0042670	Retinal cone cell differentiation	3	3	0.00011
GO:0007507	Heart development	139	17	0.000359
GO:0050793	Regulation of development	335	31	0.000369
GO:0050767	Regulation of neurogenesis	91	13	0.000387
GO:0048514	Blood vessel morphogenesis	264	25	0.000947
GO:0007275	Development	3662	211	0.0014
GO:0045665	Negative regulation of neuron differentiation	12	4	0.00191
GO:0048708	Astrocyte differentiation	6	3	0.00197
GO:0048710	Regulation of astrocyte differentiation	6	3	0.00197
GO:0042483	Negative regulation of odontogenesis	2	2	0.0023
GO:0042489	Negative regulation of odontogenesis (sensu Vertebrata)	2	2	0.0023
GO:0001525	Angiogenesis	240	22	0.00279
GO:0007517	Muscle development	256	23	0.00293
GO:0043353	Enucleate erythrocyte differentiation	7	3	0.00333
GO:0048592	Eye morphogenesis	42	7	0.00353
GO:0045995	Regulation of embryonic development	14	4	0.00357
GO:0008544	Epidermis development	174	17	0.00426
GO:0007498	Mesoderm development	58	8	0.00619
GO:0042481	Regulation of odontogenesis	3	2	0.00667
GO:0007398	Ectoderm development	197	18	0.00668
GO:0030033	Microvillus biogenesis	9	3	0.00743
GO:0050768	Negative regulation of neurogenesis	37	6	0.00777
GO:0007530	**Sex determination**	27	5	0.00839
GO:0050770	Regulation of axonogenesis	38	6	0.00887

'Genes in Category' represents number of genes included in the GO category; 'Genes in List in Category' represents number of altered genes in the GO category. GO terms are ranked by *p* value. An arbitrary significant GO term is shown in bold.

Table II-3 Gene Ontology Molecular Function terms statistically significantly altered in the patient ($p < 0.001$)

GO ID	GO Term	Genes in Category	Genes in List in Category	*p*-value
Upregulated in the patient				
GO:0047115	Trans-1,2-dihydrobenzene-1,2-diol dehydrogenase activity	6	5	0.00000136
GO:0016564	**Transcriptional repressor activity**	361	38	0.00000362
GO:0003714	**Transcription corepressor activity**	179	24	0.00000411
GO:0004883	Glucocorticoid receptor activity	4	4	0.000005
GO:0008195	Phosphatidate phosphatase activity	8	5	0.0000117
GO:0005201	Extracellular matrix structural constituent	143	20	0.0000136
GO:0030106	MHC class I receptor activity	36	9	0.0000341
GO:0004857	Enzyme inhibitor activity	318	32	0.0000508
GO:0047026	3-alpha-hydroxysteroid dehydrogenase (A-specific) activity	3	3	0.000106
GO:0004033	Aldo-keto reductase activity	19	6	0.000177
GO:0004866	Endopeptidase inhibitor activity	160	19	0.000209
GO:0030414	Protease inhibitor activity	161	19	0.000227
GO:0008201	Heparin binding	136	17	0.00024
GO:0003712	**Transcription cofactor activity**	501	41	0.000484
GO:0004030	Aldehyde dehydrogenase [NAD(P)+] activity	10	4	0.000835
GO:0008134	**Transcription factor binding**	683	51	0.000893
GO:0016628	Oxidoreductase activity, acting on the CH-CH group of donors, NAD or NADP as acceptor	25	6	0.000906
Downregulated in the patient				
GO:0016728	Oxidoreductase activity, acting on CH2 groups, disulfide as acceptor	4	4	0.00000289
GO:0004748	Ribonucleoside-diphosphate reductase activity	4	4	0.00000289
GO:0047485	Protein N-terminus binding	85	14	0.00000928
GO:0005515	Protein binding	9920	466	0.0000111
GO:0003918	DNA topoisomerase (ATP-hydrolyzing) activity	5	4	0.000014
GO:0016860	Intramolecular oxidoreductase activity	57	11	0.000018
GO:0016725	Oxidoreductase activity, acting on CH2 groups	7	4	0.0000914
GO:0003824	Catalytic activity	6300	306	0.000211
GO:0015038	Glutathione disulfide oxidoreductase activity	4	3	0.000272
GO:0048027	mRNA 5'-UTR binding	4	3	0.000272
GO:0015037	Peptide disulfide oxidoreductase activity	4	3	0.000272
GO:0004667	Prostaglandin-D synthase activity	4	3	0.000272

(Continued on next page)

Table II-3 (Continued)

GO ID	GO Term	Genes in Category	Genes in List in Category	*p*-value
GO:0016655	Oxidoreductase activity, acting on NADH or NADPH, quinone or similar compound as acceptor	43	8	0.000327
GO:0016862	Intramolecular oxidoreductase activity, interconverting keto- and enol-groups	17	5	0.000484
GO:0046870	Cadmium ion binding	10	4	0.000496
GO:0003916	DNA topoisomerase activity	10	4	0.000496

'Genes in Category' represents number of genes included in the GO category; 'Genes in List in Category' represents number of altered genes in the GO category. GO terms were ranked by *p* value. Arbitrary significant GO terms are shown in bold.

Table II-4 Gene Ontology Cellular Component terms statistically significantly altered in the patient ($p < 0.001$)

GO ID	GO Term	Genes in Category	Genes in List in Category	*p*-value
Upregulated in the patient				
GO:0005581	Collagen	76	15	0.00000232
GO:0005578	Extracellular matrix (sensu Metazoa)	748	62	0.0000135
GO:0042611	MHC protein complex	78	14	0.0000159
GO:0031012	Extracellular matrix	757	62	0.0000194
GO:0005583	Fibrillar collagen	22	7	0.0000481
GO:0005586	Collagen type III	3	3	0.000106
GO:0016011	Dystroglycan complex	12	5	0.000142
GO:0001527	Microfibril	7	4	0.000157
GO:0016012	Sarcoglycan complex	8	4	0.000302
GO:0001772	Immunological synapse	102	14	0.000322
GO:0042612	**MHC class I protein complex**	50	9	0.000507
GO:0005604	Basement membrane	122	15	0.000663
GO:0005605	Basal lamina	52	9	0.000684
Downregulated in the patient				
GO:0005622	Intracellular	13108	580	0.000000182
GO:0005737	Cytoplasm	8736	412	0.000000594
GO:0005635	Nuclear membrane	313	31	0.00000308
GO:0005694	Chromosome	505	42	0.00000569
GO:0015630	Microtubule cytoskeleton	547	44	0.00000799
GO:0005874	Microtubule	305	29	0.000014
GO:0005654	Nucleoplasm	1096	70	0.0000594
GO:0043232	Intracellular non-membrane-bound organelle	2299	127	0.0000646
GO:0043228	Non-membrane-bound organelle	2299	127	0.0000646
GO:0005819	Spindle	120	15	0.0000841
GO:0043229	Intracellular organelle	10447	464	0.000108
GO:0043226	Organelle	10447	464	0.000108
GO:0016235	Aggresome	4	3	0.000244
GO:0043231	Intracellular membrane-bound organelle	9339	417	0.000318
GO:0043227	Membrane-bound organelle	9339	417	0.000318
GO:0000777	Condensed chromosome kinetochore	36	7	0.000468
GO:0030496	Midbody	18	5	0.000548
GO:0000779	**Condensed chromosome, pericentric region**	38	7	0.00066
GO:0000775	**Chromosome, pericentric region**	103	12	0.000802

'Genes in Category' represents number of genes included in the GO category; 'Genes in List in Category' represents number of altered genes in the GO category. GO terms were ranked by *p* value. Arbitrary significant GO terms are shown in bold.

CONCLUSION

In the mammalian sex differentiation, two steps have been distinguished [Jost *et al*., 1973]: the first step from the chromosomes to the formation of the gonads (controlled by sex-determining genes) and the second step from the gonads to the phenotype (by production of gonadal hormones). DSD occur by disorders in these processes. It has been reported that DSD occur both in humans and in domestic animals—goats, llama, sheep, pigs, dogs, cats, rabbits, and horses. The identification of *SRY* in 1990 was the first crucial step towards a general understanding of sex determination or differentiation. Now, after 20 years of research in this field, analysis of individuals with DSD has led to the identification of several new genes in the sex-determination cascade. Furthermore, substantial information has been obtained by studying the following model animals with DSD—such as (1) C57BL/6J (B6) mice carrying a Y chromosome of *Mus domesticus poschiavinus* origin (XY^{POS}), which develop either as females with exclusively ovarian tissue or as true hermaphrodites with ovarian and testicular tissues [Eicher *et al*., 1996], and (2) *M33* (homologous to the *Drosophila* polycomb genes) mutant mice showing XY sex reversal [Katoh-Fukui *et al*., 1998]. However, many of the interactions between these sex-determining genes/proteins remain to be elucidated, and certainly, other sex-determining genes and proteins remain to be identified.

This study involved a 17-year-old XY female. The results of clinical analysis indicated that *SRY* mRNA, which essentially reaches peak levels at 44 days of gestation in the testis cord, was highly expressed not only in her gonads but also in her skin fibroblasts. In addition, alterations—at the gene and/or pathway levels—in the expression of numerous genes acting at the developmental stage (including sex-determining genes) were detected, and these alterations may have persisted as a vestige of anomalies of sex differentiation that presumably began in the fetal period.

In normal ontogenetic development, *SRY* expression, involved in the first step of sex differentiation in males (depending on the coordinated expression of specific genes), is regulated in a strictly spatio-temporal manner. Therefore, delayed *Sry* expression [Bullejos and Koopman, 2005]; insufficient *Sry* expression [Albrecht *et al*., 2003; Washburn *et al*., 2001]; and delayed downregulation of the SRY protein, which must be downregulated in close association with testis cord organization [Taketo *et al*., 2005], are associated with sex reversal in mice. The current finding of prolonged activity of *SRY* strongly suggests that accurate regulation of *SRY* mRNA expression is essential for normal sex differentiation. A disturbance in *SRY* regulation might inhibit diversion into the male sex-determination cascade, thereby tilting the sexual fate toward the female sex-determination cascade—the default pathway in mammals—even in the case of normal SRY translation.

Recently, epigenetics has been considered to be a novel area of research and is expected to be extensively investigated. The alteration of *SRY* expression in the case of the XY female in my study suggested a possible mechanism for the activation of *SRY* expression via histone H3 acetylation. A high rate of morphological and histopathological abnormalities has been reported in cloned animals [Wells *et al*., 1999; Renard *et al*., 1999; Ono *et al*., 2001], and epigenetic instability is thought to be causally related to these various abnormalities [Humpherys *et al*., 2001]. From this standpoint, my findings provide a unique opportunity to understand the association between abnormalities of sex determination or differentiation and epigenetic abnormalities.

The current study provides a great deal of thought-provoking information, which is expected to inspire future research in this field. Further studies on DSD in humans will contribute to the identification of unknown genes and to a more comprehensive understanding of mechanisms that underlie mammalian sex determination.

SUMMARY IN JAPANESE

生物が遺伝的多様性を獲得するために構築した有性生殖は，次世代に遺伝情報を受け継ぐシステムであり，性分化は極めて重要な個体発生の分化過程あるいは生命活動と考えられる．ヒトをはじめとする哺乳類の性分化は，雄型と雌型の 2 通りのプログラムが用意されているわけではなく，基本型は雌型であり雄型は雌型から誘導される形で分化するとされる．すなわち，未分化性腺を精巣に誘導する精巣決定遺伝子である Y 染色体上の SRY（sex determining region of the Y chromosome）遺伝子が働く場合のみ雄に分化し，働かない場合は自動的に雌に分化する．SRY 遺伝子の同定により性分化機構は速やかに解明されるものと思われたが，SRY 遺伝子の突然変異や欠失，転座では説明のつかない性逆転の例が見つかるにつれ，1 つの遺伝子によって制御されるほど単純なものではないことが明らかとなった．近年の分子生物学的手法の発展ならびに性分化疾患の症例解析から多くの性分化関連遺伝子が明らかにされ，未分化性腺で時空間的に作用する性分化関連遺伝子群の存在が明らかになるにつれて，性腺発生の複雑さが再認識され，性の分化とその破綻に関する研究分野は，SRY 遺伝子の同定から 20 年経った現在も未だ端緒の域にあるとさえ言えるかも知れない．たとえば，XY 染色体型表現型女性（XY 型性逆転）症例において原因（SRY 遺伝子異常）が明らかとなっているのは 15%程度にすぎず，XY 型性逆転発症の分子基盤は，依然として不明な点が多く残されている．

ゲノムの塩基配列の変化を伴わず細胞分裂後も継承される遺伝子機能の変化とそのしくみ（およびその学問領域）であるエピジェネティクスは，発生・分化に伴う細胞の表現型の情報記憶に中心的な役割を担っていることが明らかとなり，近年，大きく注目されている．エピジェネティクスは，DNA のメチル化とクロマチンのリモデリングによる遺伝子発現変化と捉えられており，エピジェネティクスに異常を来たすと発生分化に障害が生じる．遺伝子発現の増減を決めているのは転写因子の働きであるが，遺伝子のオン／オフには DNA メチル化とクロマチン構造変換が関与しており，性分化異常の

分子基盤解明においても，従来の細胞遺伝学的・分子生物学的解析に加え，エピゲノム解析の展開が期待される．また，性分化とその破綻の複雑な発症機構においては，分子背景の網羅的解析が必須であると考えられる．近年，DNA マイクロアレイ技術とバイオインフォマティックスの発展により，数万の遺伝子発現を網羅的に解析し，既存の遺伝子データベースと照らし合わせ，新たな生物学的解釈を見出すことが可能となった．

本論文では，原因不明の XY 型性逆転に関して，従来の細胞分子形態学的解析に加え，新たに SRY 遺伝子の発現制御機構とエピジェネティクスとの関連ならびに発現変動を示す遺伝子の相互作用や全体の協調的な挙動，転写因子やパスウェイレベルでの発現変動に注目し，性逆転を引き起こした分子基盤を明らかにするとともに，新たな雄化破綻機構を提案することを試みた．

患者は，17 歳の戸籍上女性で，原発性無月経を主訴として近医を受診した．体毛はなく，発育不良の乳房ならびにホルモン剤に反応し消退出血が起こる機能性子宮を有していた．ゴナドトロピンは高値，性ステロイドホルモンは極めて低値であった．出生時の体重は 1,910g（妊娠 39 週）の過小児で，鼠径ヘルニアが認められ，2 歳時にはヘルニア手術を施行されている．その他，膜性腎症の既往歴があり，染色体検査で Y 染色体が確認され，泌尿生殖器系の発生異常を含めた性分化疾患が疑われた．

第 I 章では，FISH 解析も含めた詳細な細胞遺伝学的検索で 46,X,inv(Y)(p11.2q11.2)，すなわち，Y 染色体の腕間逆位が明らかとなったが，SRY 遺伝子の転座はなく，また Y 染色体に欠失も認められなかった．一方，SRY 遺伝子に 613C→T への置換が明らかとなったが，アミノ酸変異の無い SNP であり，さらに SRY 蛋白陽性の索状性腺であったことから，SRY 遺伝子および転写翻訳機能に異常はないと考えられた．

SRY 遺伝子の発現状態を検討した結果，患者の性腺および皮膚線維芽細胞で SRY mRNA の過剰発現が認められ，一方，正常男性由来皮膚線維芽細胞ではほとんど発現がみられず，時空間的に発現制御されるべき SRY 遺伝子の発現調節異常が疑われた．SRY 遺伝子領域におけるヒストン H3 のアセチル化状態を解析したところ，正常男性と比較して患者では，遺伝子発現を活性化するアセチル化ヒストンの割合が極めて高いことが

明らかとなり，mRNA の高発現が裏付けられた．さらに GeneChip を用いたマイクロアレイ解析では，ヒストンアセチル化酵素（histone acetyl transferase: HAT）を活性化する PCAF（p300/CBP-associated factor）遺伝子の高発現が患者に認められた．転写誘導とよく相関するヒストン修飾として，ヒストン H3 K9 と K14 のアセチル化が知られているが，PCAF は主に K14 をアセチル化修飾することが明らかとなっている．PCAF 遺伝子の高発現がクロマチン・リモデリング因子をリクルートし，本来，サイレンシング状態にあるべき SRY 遺伝子を活性化しているものと推察された．

遺伝子カスケードの雄化スイッチとして機能する SRY 遺伝子は，正常な個体発生において，その発現時期および発現量が巧妙に制御されている．この SRY 遺伝子の発現調節異常は，下流カスケードを担う遺伝子群の時空間的な発現をも攪乱すると考えられ，本患者の場合，おそらくは胎児期において，SRY 遺伝子の高発現を主とする発現調節異常が存在したものと推測された．さらには，従来，原因不明であった XY 型性逆転症例において，エピジェネティック異常が原因の一つである可能性が考えられ，多様な性分化疾患の発症機構において，SRY 遺伝子のエピジェネティックな変異が関与することが強く示唆された．

第 II 章では，患者および正常男性由来皮膚線維芽細胞において，マイクロアレイ解析で得られた遺伝子発現プロファイルの差異を検討したところ，22,283 個の遺伝子のうち，1,820 個の遺伝子に発現変化が認められた．それらの遺伝子の生物学的解釈を行うため，多数の遺伝子の発現データから特徴的な発現パターンを発見し，生物学的意義を見出すデータマイニング技術により Gene Ontology（GO）解析を行った．GO は、遺伝子産物がどのような生物学的プロセスに関与するか，どのような細胞構成要素に含まれているか，どのような分子機能を持っているかを記述するための体系化された用語（GO term）とその用語間の関係を定義したものであり，GO term 間の関係は“Biological process”，“Cellular component”，“Molecular function”の 3 つの GO term を最上位とする階層構造で表現される．本解析により，“Biological process”において比較的上位階層である GO term “development”に含まれる遺伝子群のアップレギュレーションが明らかとな

り，さらにその下層に GO term “sex determination”が見出された．また，パスウェイ解析においては，胚発生時に重要な機能を果たす“TGF-β signaling pathway” や“Wnt signaling pathway” が変動していた．すなわち，発生や形態形成に関わる遺伝子群の発現変化や胚発生に関わるパスウェイの変化が認められ，それらは胎児期における性分化異常の原因の痕跡として残っているものと考えられた．また，ヒトの性分化疾患に関し，バイオインフォマティックスを駆使した解析はこれまでになく，今回得られた知見から，データマイニング法は原因不明の XY 型性逆転の発症原因を研究する上で有用なツールになり得ると考えられた．

以上より，胎児期における性分化異常の痕跡は，SRY 遺伝子をはじめとする性分化関連遺伝子の発現異常として生後も性腺や線維芽細胞等の体細胞に残っていることが示され，さらにその痕跡はパスウェイレベルで見出されることが明らかとなった．この結果は，性分化疾患の発症機構を解析する上で，新たな基礎的知見を提起するものであると考えられる．また，正常な雄への性分化には，SRY 発現が適切な時期に適切な量存在することが極めて重要であり，たとえ正常な SRY 蛋白に翻訳されたとしても，不適切な時期に作られた転写因子（蛋白質）によるその発現調節異常は下流の遺伝子カスケードを攪乱し，雄化が障害され，その結果，哺乳動物の原型としての雌型へ誘導されることが推察された．さらに本症例で得られた解析結果は，SRY 遺伝子領域のヒストンアセチル化異常というエピジェネティックな関与を示唆するとともに多くの重要な基礎データを提供するものであり，これまで原因不明であった XY 型性逆転の発症機構の解明に新たな展開をもたらすものと期待される．

ACKNOWLEDGEMENTS

I would like to acknowledge the following people for assisting me in this study. My deepest thanks go to Prof. Nobuhiko HOSHI (Department of Animal Science, Graduate School of Agricultural Science, Kobe University), my supervisor, for his invaluable input during this research and for giving me this opportunity to study. I would also like to thank Prof. Hiroshi KITAGAWA (Department of Animal Science, Graduate School of Agricultural Science, Kobe University) for expert advice. I greatly appreciate the help of Prof. Masashi MIYAKE (Department of Animal Science, Graduate School of Agricultural Science, Kobe University), who read this thesis and helped to improve the writing. I also express my sincere gratitude to Associate Prof. Yoshiaki TABUCHI, Assistant Prof. Ichiro TAKASAKI, Prof. Takashi KONDO (University of Toyama) and Prof. Teruo SUGAWARA (Otaru University of Commerce) for their excellent technical assistance. I am deeply grateful to Prof. Noriaki SAKURAGI and Dr. Yasuhiko EBINA (Department of Obstetrics and Gynecology, Hokkaido University) for contributing dermal and gonadal samples and clinical information. I thank Prof. Fumio HAYASHI (Department of Biology, Faculty of Science, Kobe University) for his continuous encouragement. Special thanks should also go to Dr. Katsuhiko WARITA, who helped me complete this research. Finally, I thank Assistant Prof. Toshifumi YOKOYAMA and other members of the Laboratory for Functional Morphology, Department of Animal Science, Graduate School of Agricultural Science, Kobe University for their invaluable help during the course of the study. The continuing support and encouragement of all these experts and collaborators have been instrumental in the progress I have made in my work to date. I offer them all my heartfelt gratitude.

REFERENCES

Albrecht KH, Young M, Washburn LL, Eicher EM (2003) *Sry* expression level and protein isoform differences play a role in abnormal testis development in C57BL/6J mice carrying certain *Sry* alleles. *Genetics* **164**: 277–288.

Ashburner M, Ball CA, Blake JA, Botstein D, Butler H, Cherry JM, Davis AP, Dolinski K, Dwight SS, Eppig JT, Harris MA, Hill DP, Issel-Tarver L, Kasarskis A, Lewis S, Matese JC, Richardson JE, Ringwald M, Rubin GM, Sherlock G (2000) Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. *Nat Genet.* **25**: 25–29.

Barsky A, Gardy JL, Hancock RE, Munzner T (2007) Cerebral: a Cytoscape plugin for layout of and interaction with biological networks using subcellular localization annotation. *Bioinformatics* **23**: 1040–1042.

Biason-Lauber A, Konrad D, Meyer M, deBeaufort C, Schoenle EJ (2009) Ovaries and female phenotype in a girl with 46,XY karyotype and mutations in the *CBX2* gene. *Am J H*um *Genet* **84**: 658–663.

Boyes J, Bird A (1992) Repression of genes by DNA methylation depends on CpG density and promoter strength: evidence for involvement of a methyl-CpG binding protein. *EMBO J* **11**: 327–333.

Buckle V, Mondello C, Darling S, Craig IW, Goodfellow PN (1985) Homologous expressed genes in the human sex chromosome pairing region. *Nature* **317**: 739–741.

Bullejos M, Koopman P (2005) Delayed Sry and Sox9 expression in developing mouse gonads underlies B6-Y^{DOM} sex reversal. *Develop Biol* **278**: 473–481.

Choi EY, Park WS, Jung KC, Kim SH, Kim YY, Lee WJ, Park SH (1998) Engagement of CD99 induces up-regulation of TCR and MHC class I and II molecules on the surface of human thymocytes. *J Immunol* **161**: 749–754.

Clépet C, Schater AJ, Sinclair AH, Palmer MS, Lovell-Badge R, Goodfellow PN (1993) The human *SRY* transcript. *Hum Mol Genet* **2**: 2007–2012.

Corbo RM, Ulizzi L, Piombo L, Martinez-Labarga C, De Stefano GF, Scacchi R (2007) Estrogen receptor alpha polymorphisms and fertility in populations with different reproductive patterns. *Mol Hum Reprod* **13**: 537–540.

Cost NG, Ludwig AT, Wilcox DT, Rakheja D, Steinberg SJ, Baker LA (2009) A novel *SOX9* mutation, 972delC, causes 46,XY sex-reversed campomelic dysplasia with nephrocalcinosis, urolithiasis, and dysgerminoma. *J Pediatr Surg* **44**: 451–454.

Cui Q, Ma Y, Jaramillo M, Bari H, Awan A, Yang S, Zhang S, Liu L, Lu M, O'Connor-McCourt M, Purisima EO, Wang E (2007) A map of human cancer signaling. *Mol Syst Biol* **3**: 152.

Dahlquist KD, Salomonis N, Vranizan K, Lawlor SC, Conklin BR (2002) GenMAPP, a new tool for viewing and analyzing microarray data on biological pathways. *Nat Genet* **31**: 19–20.

Eicher EM, Washburn LL (1986) Genetic control of primary sex determination in mice. *Annu Rev Genet* **20**: 327–360.

Eicher EM, Washburn LL, Schork NJ, Lee BK, Shown EP, Xu X, Dredge RD, Pringle MJ, Page DC (1996) Sex-determining genes on mouse autosomes identified by linkage analysis of C57BL/6J-YPOS sex reversal. *Nat Genet* **14**: 206–209.

Ford CE, Jones KW, Polani PE, De Almeida JC, Briggs JH (1959) A sex-chromosome anomaly in a case of gonadal dysgenesis (Tutner's syndrome). *Lancet* **1**: 711–713.

Franzén P, ten Dijke P, Ichijo H, Yamashita H, Schulz P, Heldin CH, Miyazono K (1993) Cloning of a TGF-β type I receptor that forms a heteromeric complex with the TGF-β type II receptor. *Cell* **75**: 681–692.

Fujimoto S, Hayashi H (1983) [Sex differentiation abnormalities.] Clin Gynecol Obstet **37**: 621–631 (In Japanese).

Gimelli G, Giorda R, Beri S, Gimelli S, Zuffardi O (2006) A 46,X,inv(Y) young woman with gonadal dysgenesis and gonadoblastoma: cytogenetics, molecular, and methylation studies. *Am J Med Genet A* **140A**: 40–45.

Giuffrè, M, Sammarco P, Fabiano C, Giardina F, Lunetta F, Corsello G (2004) Identification of a new nonsense mutation (Tyr129Stop) of the SRY gene in a newborn infant with XY sex-reversal. *Am J Med Genet* **128A**: 46–47.

Grunstein M (1997) Histone acetylation in chromatin structure and transcription. *Nature* **389**: 349–352.

Gruenbaum Y, Stein R, Cedar H, Razin A (1981) Methylation of CpG sequences in eukaryotic DNA. *FEBS Lett* **124**: 67–71.

Hacker A, Capel B, Goodfellow P, Lovell-Badge (1995) Expression of Sry, the mouse sex determining gene. *Development* **121**: 1603–1614.

Hanley NA, Hagan DM, Clement-Jones M, Ball SG, Strachan T, Salas-Cortés L, McElreavey K, Lindsay S, Robson S, Bullen P, Ostrer H, Wilson DI (2000) *SRY*, *SOX9*, and *DAX1* expression patterns during human sex determination and gonadal development. *Mech Dev* **91**: 403–407.

Hawkins JR, Taylor A, Berta P, Levilliers J, Van der Auwera B, Goodfellow PN (1992a) Mutational analysis of SRY: nonsense and missense mutations in XY sex reversal. *Hum Genet* **88**: 471–474.

Hawkins JR, Taylor A, Goodfellow PN, Migeon CJ, Smith KD, Berkovitz GD (1992b) Evidence for increased prevalence of SRY mutations in XY females with complete rather than partial gonadal dysgenesis. *Am J Hum Genet* **51**: 979–984.

Hoshi N, Fujita M, Mikuni M, Fujino T, Okuyama K, Handa Y, Yamada H, Sagawa T, Hareyama H, Nakahori Y, Fujieda K, Kant JA, Nagashima K, Fujimoto S (1998a) Seminoma in a postmenopausal woman with a Y;15 translocation in peripheral blood lymphocytes and a t(Y;15)/45,X Turner mosaic pattern in skin fibroblasts. *J Med Genet* **35**: 852–856.

Hoshi N, Tonoki H, Handa Y, Fujino T, Okuyama K, Koga Y, Matsumoto Y, Yamada T, Yamada H, Kishida T, Sagawa T, Fujieda K, Nakahori Y, Kant JA, Fujimoto S (1998b) Prenatal identification of mos45,X/46,X+mar in a normal male baby by cytogenetic and molecular analysis. *Prenat Diagn* **18**: 1316–1322.

Humpherys D, Eggan K, Akutsu H, Hochedlinger K, Rideout WM 3rd, Biniszkiewicz D, Yanagimachi R, Jaenisch R (2001) Epigenetic instability in ES cells and cloned mice. *Science* **293**: 95–97.

Iida T, Nakahori Y, Komaki R, Mori E, Hayashi N, Tsutsumi O, Taketani Y, Nakagome Y (1994) A novel nonsense mutation in the HMG box of the SRY gene in a patient with XY sex reversal. *Hum Mol Genet* **3**: 1437–1438.

Imai A, Takagi A, Tamaya T (1999) A novel sex-determining region on Y (SRY) missense mutation identified in a 46,XY female and also in the father. *Endocr J* **46**: 735–739.

Jacobs PA, Strong JA (1959) A case of human intersexuality having a possible XXY sex-determining mechanism. *Nature* **183**: 302–303.

Jäger RJ, Anvret M, Hall K, Scherer G (1990) A human XY female with a frame shift mutation in the candidate testis-determining gene *SRY. Nature* **348**: 452–454.

Jeske YW, Bowles J, Greenfield A, Koopman P (1995) Expression of a linear Sry transcript in the mouse genital ridge. *Nat Genet* **10**: 480–482.

Johnson DW, Qumsiyeh M, Benkhalifa M, Marchuk DA (1995) Assignment of human transforming growth factor-β type I and type III receptor genes (TGFBR1 and TGFBR3) to 9q33-q34 and 1p32-p33, respectively. *Genomics* **28**: 356–357.

Jost A, Vigier B, Prépin J, Perchellet JP (1973) Studies on sex differentiation in mammals. *Recent Prog Horm Res* **29**: 1–41.

Kadonaga JT (1998) Eukaryotic transcription: an interlaced network of transcription factors and chromatin-modifying machines. *Cell* **92**: 307–313.

Katoh-Fukui Y, Tsuchiya R, Shiroishi T, Nakahara Y, Hashimoto N, Noguchi K, Higashinakagawa T (1998) Male-to-female sex reversal in M33 mutant mice. *Nature* **393**: 688–692.

Kellermayer R, Halvax L, Czakó M, Shahid M, Dhillon VS, Husain SA, Süle N, Gömöri E, Mammel M, Kosztolányi G (2005) A novel frame shift mutation in the HMG box of the *SRY* gene in a patient with complete 46,XY pure gonadal dysgenesis. *Diagn Mol Pathol* **14**: 159–163.

Kim SY, Volsky DJ (2005) PAGE: parametric analysis of gene set enrichment. *BMC Bioinformatics* **6**: 144.

Klattig J, Sierig R, Kruspe D, Makki MS, Englert C (2007) WT1-mediated gene regulation in early urogenital ridge development. *Sex Dev* **1**: 238–254.

Knower KC, Sim H, McClive PJ, Bowles J, Koopman P, Sinclair AH, Harley VR (2007) Characterisation of urogenital ridge gene expression in the human embryonal carcinoma cell line NT2/D1. *Sex Dev* **1**: 114–126.

Kojima Y, Hayashi Y, Mizuno K, Sasaki S, Fukui Y, Koopman P, Morohashi K, Kohri K (2008) Up-regulation of SOX9 in human *sex-determining region on the Y chromosome (SRY)*-negative XX males. *Clin Endocrinol* **68**: 791–799.

Koopman P, Münsterberg A, Capel B, Vivian N, Lovell-Badge R (1990) Expression of a candidate sex-determining gene during mouse testis differentiation. *Nature* **348**: 450–452.

Koopman P, Gubbary J, Vivian N, Goodfellow P, Lovell-Badge R (1991) Male development of chromosomally female mice transgenic for *Sry*. *Nature* **351**: 117–121.

Krishna Murthy DS, Murthy SK, Patel JK, Banker GN, Shah VC (1989) Inherited pericentric inversion of Y-chromosome with trisomy 21. *Ann Genet* **32**: 47–51.

Kurebayashi J, Otsuki T, Kunisue H, Tanaka K, Yamamoto S, Sonoo H (2000) Expression levels of estrogen receptor-α, estrogen receptor-β, coactivators, and corepressors in breast cancer. *Clin Cancer Res* **6**: 512–518.

Li B, Zhang W, Chan G, Jancso-Radek A, Liu S, Weiss MA (2001) Human sex reversal due to impaired nuclear localization of SRY. A clinical correlation. *J Biol Chem* **276**: 46480–46484.

Lim HN, Freestone SH, Romero D, Kwok C HughesIA, Hawkins JR (1998) Candidate genes in complete and partial XY sex reversal: mutation analysis of SRY, SRY-related genes and FTZ-F1. *Mol Cell Endocrinol* **140**: 51–58.

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods* **25**: 402–408.

Marchina E, Gambera A, Spinelli E, Clerici P, Scagliola P, Sartori E, Barlati S (2009) Identification of a new mutation in the *SRY* gene in a 46,XY woman with Swyer syndrome. *Fertil Steril* **91**: 932.e7–932.e11.

McElreavey K, Vilain E, Abbas N, Herskowitz I, Fellous M (1993) A regulatory cascade hypothesis for mammalian sex determination: SRY represses a negative regulator of male development. *Proc Natl Acad Sci USA* **90**: 3368–3372.

Modi D, Shah C, Sachdeva G, Gadkar S, Bhartiya D, Puri C (2005) Ontogeny and cellular localization of SRY transcripts in the human testes and its detection in spermatozoa. *Reproduction* **130**: 603–613.

Müller J, Schwartz M, Skakkebæk NE (1992) Analysis of the sex-determining region of the Y chromosome (SRY) in sex reversed patient: point-mutation in SRY causing sex-reversion in a 46,XY female. *J Clin Endocrinol Metabol* **75**: 331–333.

Nakahori Y, Tamura T, Nagafuchi S, Fujieda K, Minowada S, Fukutani K, Fuse H, Hayashi K, Kuroki Y, Fukushima Y, Agematsu K, Kuno T, Kaneko S, Yamada K, Kitagawa T, Nonomura M, Fukuda S, Kusano M, Onigata S, Hibi I, Nakagome Y (1991) Molecular cloning and mapping of 10 new probes on the human Y chromosome. *Genomics* **9**: 765–769.

Nishino K, Hattori N, Tanaka S, Shiota K (2004) DNA methylation mediated control of *Sry* gene expression in mouse gonadal development. *J Biol Chem* **279**: 22306–22313.

Ng HH, Bird A (2000) Histone deacetylases: silencers for hire. *Trends Biochem Sci* **25**: 121–126.

Okuhara K, Tajima T, Nakae J, Fujieda K (2000) A novel missense mutation in the HMG box region of the SRY gene in a Japanese patient with an XY sex reversal. *J Hum Genet* **45**: 112–114.

Olesen C, Hansen C, Bendsen E, Byskov AG, Schwinger E, Lopez-Pajares I, Jensen PK, Kristoffersson U, Schubert R, Van Assche E, Wahlstroem J, Lespinasse J, Tommerup N (2001) Identification of human candidate genes for male infertility by digital differential display. *Mol Hum Reprod* **7**: 11–20.

Ono Y, Shimozawa N, Ito M, Kono T (2001) Cloned mice from fetal fibroblast cells arrested at metaphase by a serial nuclear transfer. *Biol Reprod* **64**: 44–50.

Pajares G, Balseiro A, Pérez-Pardal L, Gamarra JA, Monteagudo LV, Goyache F, Royo LJ (2009) Sry-negative XX true hermaphroditism in a roe deer. *Anim Reprod Sci* **112**: 190–197.

Piccione M, Maresi E, Zollino M, Sanfilippo C, Seminara L, Neri G, Corsello G (2008) Sex reversal from functional disomy of Xp: prenatal and post-mortem findings. *Am J Med Genet A* **146A**: 2681–2687.

Pires-daSilva A, Sommer RJ (2003) The evolution of signalling pathways in animal development. *Nat Rev Genet* **4**: 39–49.

Pontiggia A, Rimini R, Harley VR, Goodfellow PN, Lovell-Badge R, Bianchi ME (1994) Sex-reversing mutations affect the architecture of SRY–DNA complexes. *EMBO J* **13**: 6115–6124.

Radpour R, Falah M, Aslani A, Zhong XY, Saleki A (2009) Identification of a critical novel mutation in the exon 1 of androgen receptor gene in 2 brothers with complete androgen insensitivity syndrome. *J Androl* **30**: 230–232.

Razin A, Webb C, Szyf M, Yisraeli J, Rosenthal A, Naveh-Many T, Sciaky-Gallili N, Cedar H (1984) Variations in DNA methylation during mouse cell differentiation in vivo and in vitro. *Proc Natl Acad Sci USA*. **81**: 2275–2279.

Renard JP, Chastant S, Chesné P, Richard C, Marchal J, Cordonnier N, Chavatte P, Vignon X (1999) Lymphoid hypoplasia and somatic cloning. *Lancet* **353**: 1489–1491.

Robinson A, Linden MG (1993) Ambiguous genitalia and hermaphroditism. In: *Clinical Genetics Handbook,* 2nd edn. Blackwell Scientific, Boston, U.S.A., pp309–315.

Salas-Cortés L, Jaubert F, Barbaux S, Nessmann C, Bono MR, Fellous M, McElreavey K, Rosemblatt M (1999) The human SRY protein is present in fetal and adult Sertoli cells and germ cells. *Int J Dev Biol* **43**: 135–140.

Salas-Cortés L, Jaubert F, Bono MR, Fellous M, Rosemblatt M (2001) Expression of the human SRY protein during development in normal male gonadal and sex-reversed tissues. *J Exp Zool* **290**: 607–615.

Salas-Cortés L, Jaubert F, Nihoul-Feketé C, Brauner R, Rosemblatt M, Fellous M (2000) SRY protein is expressed in ovotestis and streak gonads from human sex-reversal. *Cytogenet Cell Genet* **91**: 212–216.

Schmid M (1985) Variations of content and structure of the mammalian Y chromosome. In: Sandberg AA (eds) *The Y Chromosome Part A: Basic Characteristics of the Y Chromosome.* Liss, A.R., New York, U.S.A., pp373–401.

Shahid M, Dhillion VS, Jain N, Hedau S, Diwakar S, Sachdeva P, Batra S, Das BC, Husain SA (2004) Two new novel point mutations localized upstream and downstream of the HMG box region of the *SRY* gene in three Indian 46,XY females with sex reversal and gonadal tumour formation. *Mol Hum Reprod* **10**: 521–526.

Shannon P, Markiel A, Ozier O, Baliga NS, Wang JT, Ramage D, Amin N, Schwikowski B, Ideker T (2003) Cytoscape: a software environment for integrated models of biomolecular interaction networks. *Genome Res* **13**: 2498–2504.

Sinclair AH, Berta P, Palmer MS, Hawkins JR, Griffiths BL, Smith MJ, Foster JW, Frischauf AM, Lovell-Badge R, Goodfellow PN (1990) A gene from the human sex-determining region encodes a protein with homology to a conserved DNA binding motif. *Nature* **346**: 240–244.

Slim R, Levilliers J, Lüdecke HJ, Claussen U, Nguyen VC, Gough NM, Horsthemke B, Petit C (1993) A human pseudoautosomal gene encodes the ANT3 ADP/ATP translocase and escapes X-inactivation. *Genomics* **16**: 26–33.

Smyk M, Berg JS, Pursley A, Curtis FK, Fernandez BA, Bien-Willner GA, Lupski JR, Cheung SW, Stankiewicz P (2007) Male-to-female sex reversal associated with an ~250 kb deletion upstream of *NR0B*1 (*DAX1*). *Hum Genet* **122**: 63–70.

Strahl BD, Allis CD (2000) The language of covalent histone modifications. *Nature* **403**: 41–45.

Sugawara T, Holt JA, Driscoll D, Strauss JF 3rd, Lin D, Miller WL, Patterson D, Clancy KP, Hart IM, Clark BJ, Stocco DM (1995) Human steroidogenic acute regulatory protein: functional activity in COS-1 cells, tissue-specific expression, and mapping of the structural gene to 8p11.2 and a pseudogene to chromosome 13. *Proc Natl Acad Sci USA* **92**: 4778–4782.

Swyer GI (1955) Male pseudohermaphrodism: a hitherto undescribed form. *Br Med J* **2**: 709–712.

Tabuchi Y, Takasaki I, Doi T, Ishii Y, Sakai H, Kondo T (2006) Genetic networks responsive to sodium butyrate in colonic epithelial cells. *FEBS Lett* **580**: 3035–3041.

Taketo T, Lee CH, Zhang J, Li, Y, Lee CY, Lau YF (2005) Expression of SRY proteins in both normal and sex-reversed XY fetal mouse Gonads. *Dev Dyn* **233**: 612–622.

Tóth A, Gaál M, László J (1984) Familial pericentric inversion of the Y chromosome. *Ann Genet* **27**: 60–61.

Truong LH, Kuliwaba JS, Tsangari H, Fazzalari NL (2006) Differential gene expression of bone anabolic factors and trabecular bone architectural changes in the proximal femoral shaft of primary hip osteoarthritis patients. *Arthritis Res Ther* **8**: R188.

Vaiman D, Pailhoux E (2000) Mammalian sex reversal and intersexuality: deciphering the sex-determination cascade. *Trends Genet* **16**: 488–494.

van Silfhout A, Boot AM, Dijkhuizen T, Hoek A, Nijman R, Sikkema-Raddatz B, van Ravenswaaij-Arts CM (2009) A unique 970 kb microdeletion in 9q33.3, including the *NR5A1* gene in a 46,XY female. *Eur J Med Genet* **52**: 157–160.

Washburn LL, Albrecht KH, Eicher EM (2001) C57BL/6J-*T*-associated sex reversal in mice is caused by reduced expression of a *mus domesticus Sry* allele. *Genetics* **158**: 1675–1681.

Wells DN, Misica PM, Tervit HR (1999) Production of cloned calves following nuclear transfer with cultured adult mural granulosa cells. *Biol Reprod* **60**: 996–1005.

Wilhelm D, Palmer S, Koopman P (2007) Sex determination and gonadal development in mammals. *Physiol Rev* **87**: 1–28.

Wolffe AP, Pruss D (1996) Targeting chromatin disruption: transcription regulators that acetylate histones. *Cell* **84**: 817–819.

Yang X-J, Ogryzko VV, Nishikawa J, Howard BH, Nakatani Y (1996) A p300/CBP-associated factor that competes with the adenoviral oncoprotein E1A. *Nature* **382**: 319–324.

Zeng YT, Ren ZR, Zhang ML, Huang Y, Zeng FY, Huang SZ (1993) A new de novo mutation (A113T) in HMG box of the SRY gene leads to XY gonadal dysgenesis. *J Med Genet* **30**: 655–657.

Zeuthen E, Nielsen J (1973) Pericentric Y Inversion in the General Population. *Hum Genet* **19**: 265–270.