Sry Gene Detection in Gonads of Intersex Patients Using FISH

A candidate gene for sex determination was localized within a 35 kb region of the Y chromosome immediately adjacent to the pseudoautosomal boundary. Here, we describe a study of Sry detection in the gonads of intersex patients including 5 male pseudohermaphrodite (MPH), 4 XX true hermaphrodite (TH) and each 1 mixed gonadal dysgenesis (MGD) and XX sex reversal, and in the clitoris of 3 female pseudohermaphrodite (FPH) by fluorescent in situ hybridization (FISH). Sry gene was observed in all cases of MPH and each case of MGD and sex reversal in contrast to bare expression in all cases of FPH and most of TH. To our knowledge, the Sry gene itself functions testicular determination in almost all intersex cases except true hermaphrodite, a distinguished type of intersex in a different pathogenesis. In addition, FISH might be useful to detect the translocated Sry gene and localize the signal with ease.
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Key Words : *Sex determining region of Y, Intersex, Fluorescent in situ hybridization*

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INTRODUCTION

The primary switch gene responsible for initiation and differentiation of the testis is harbored in the Y chromosome, more specifically defined as testicular determining factor (TDF) (1~4). Recently a core of master regulator, responsible for TDF, has been turned out as the name of sex determining region of Y (Sry). Sry is evolutionary conserved and Y specific among a wide range of mammals and encodes a testis-specific transcripts (5~7). Much evidence with deletion or mutation in the XY female (8~10) in comparison with translocation or gene rearrangement between Y and X, or, Y and autosome in XX male (11~14) has been documented. Among many attempts to detect the Sry gene in the intersex or tumor patients, fluorescent in situ hybridization (FISH) method has been used as a kind of powerful, sensitive tool for genetic evaluation with preservation of environmental architecture of the tissue and advantage of retrieval from archives (15~16). Pathologists can address the cytogenetic concerning of aberrent gene in the context of cellular and tissue morphology. The present study was performed to determine whether or not Sry gene existed in tissue of gonads and clitoris with various kinds of intersex including XX sex reversal and true hermaphrodite as well as pseudohermaphrodite, using the direct FISH method with Sry DNA probe.

MATERIALS AND METHODS

Case selection

Gonads and clitoris were obtained from 14 patients with intersex. The details of presentation of intersex, followed by paraffin embedding and formalin fixation. The details of presentation of intersex were as follows: 5 patients of male pseudohermaphrodite (MPH), 4 of true hermaphrodite (TH), each one of mixed gonadal dysgenesis (MGD) and sex reversal, and 3 of female pseudohermaphrodite (FPH). Gonads of all patients except 3 of FPH who underwent resection of clitoris, were examined and biopsied.

Fluorescent in situ hybridization (FISH)

Paraffin blocks from archival tissues of intersex were used, from which the tissues were cut by 5 μm section and deparaffinized in xylene and followed by rehydration to distilled water in graded alcohol (100, 90, 70%). The sections were treated with RNase at 37℃ for 20 minutes. After rinsing with Tris-buffer, the samples were subsequently treated with proteinase K (20 ng/ml) at 37 ℃ for 20 minutes. After repeated rehydration, and air-dry, the sections were soaked within the denaturation solution (pH 7.0, 70% formamide, 20% 20×SSC) at 70 ℃ for 30 minutes. After air-dry, the hybridization step

was taken. The suspension of probe in ethanol was centrifuged and the supernatant was discarded, followed by air-drying. Then, Hybrisol VII was added to the dried pellet, after a while the solution was boiled in water for 2 minutes and promptly frozen in a cubed ice tank for 1 minute. The slides were then incubated with hybridization solution overnight in a 37℃ incubator. After hybridization overnight, the slides were washed with posthybe wash (50% formamide, 10% 20×SSC) at 43℃ for 15minutes. After washing with 2×SSC at 37℃ for 8 minutes and PBS (phosphate buffered saline) at room temperature for 2 min three times, the slides were finally stained with a fluorescent DAPI for easy viewing. After detection, the results were examined with an Olympus fluorescent microscope.

DNA probe labelling

Mouse sex determining region of Y (Sry) plasmid clone (p422) were obtained from the ATCC (ATCC 63130 ; phagemid clone of mouse Sry of chromosome Y). Plasmid DNA was directly labelled with rhodamine-dUTP by the nick translation labelled method. The probe contains the 380 bp Bgl II/Pst I fragment which is non-repetitive and homologous to pY53,3, human sex-determining region. This probe has been storing in a fresh frozen state suspended in 70% ethanol.

RESULTS

Laparotomy was performed to evaluate gonadal tissue and accessory sex organs. Clinical findings were summarized in Table 1. Five MPH subjects had bilateral testes with XY chromosome ; four TH subjects had obvious uterus in either one side ; each one patient with MGD and one patient of 46 XX sex reversal also revealed testis. In MGD no obvious gonad was identified, instead rudimentary uterus was detected within the inguinal canal. Three FPH subjects had both ovaries and hence clitoridotomy was performed. All testicular tissues were composed of immature seminiferous tubules with closed lumen. Nearly all prepubertal seminiferous tubules were solely composed of vertically oriented immature Sertoli cells and interstitial cells around them without conspicuous germ cells. Both stromal and sex-cord cells were characterized by quite a few staining of bright, easily interpretable hybridization signals in MPH (Fig. 1). The testicular portions of ovotestis in TH were not different from that in MPH except for the amount of interstitium which was more in the former. The testicular part of ovotestis in TH revealed, in general, absolutely negative staining, but in one case of TH where a few weakly positive signals were demonstrated along the tubular membrane (Fig. 2). They appeared to be Sertoli cells. Gonads from patients with MGD (case 13) and

Table 1. Clinical information of surveyed cases

	Age (Yr)	Sex	Chromosome	Genitalia	Gonad	Tissue	Sry	Intersex type
1	9	F	46XY	female	Testis	testis	++	Male PH
2	4	M	46XY	ambiguous	Testis	testis	++	Male PH
3	8	M	46XY	ambiguous	Testis rud. uteri	testis	++	Male PH
4	11	F	46Xi (Yq)	female	Testis	testis	++	Male PH
5	3	F	46XY	ambiguous	Testis	testis	++	Male PH
6	11	M	46XX	ambiguous	Ovary	clitoris	−	Female PH
7	1	F	46XX	ambiguous	Ovary	clitoris	−	Female PH
8	2	F	46XX	ambiguous	Ovary	clitoris	−	Female PH
9	4	F	46XX	female	Rt : Ovotestis	ovotestis	−	True H
10	13	M	46XX	ambiguous	Lt : Ovary Ovotestis	ovotestis	−	True H
11	3	F	46XX	ambiguous	Rt : Testis Lt : Ovotestis	testis	+	True H
12	6	F	46XX	ambiguous	Rt : Ovotestis Lt : Testis	testis	−	True H
13	30	M	45XO	male	Rt : No gonad, rud. uteri Lt : Testis	testis	+	MGD
14	3/12	M	46XX	ambiguous	Testis	testis	+	Sex reversal

F : female, M : male, rud. : rudimentary
PH : pseudohermaphrodite, H : hermaphrodite, MGD : mixed gonadal dysgenesis
− : none, + : minimal expression, ++ : obvious expression

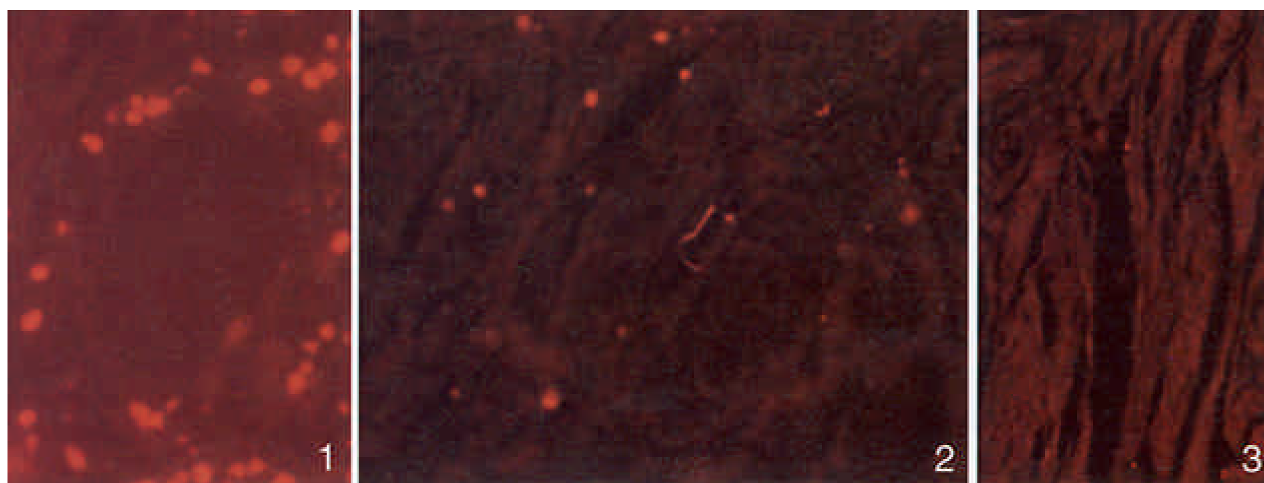

Fig. 1. Fluorescent microscopic finding of the testis in MPH. Notice brilliant signals in many cells around the tubules seeming to be sertoli cells and interstitial cells (Rhodamine, ×200).

Fig. 2. Fluorescent microscopic finding of the testis in TH. A few scattered signals are seen within tubules (Rhodamine, ×200).

Fig. 3. Fluorescent microscopic finding of the clitoris of FPH. No signals in the sphincteric muscular tissue (Rhodamine, ×200).

reversed sex (case 14) also demonstrated an immature testis composed of compact seminiferous tubules which were lined entirely by Sertoli cells and revealed a few positive signals irrespective of absence of Y chromosome. The clitoris tissues obtained from FPH patients never stained for Sry probe, thus those were used as a negative control (Fig. 3).

DISCUSSION

The subject of abnormalities of sexual differentiation is justifiably regarded as complex and confusing. Abnormalities of sexual differentiation are classified as four major categories: dismatch in chromosomal sex, errors in gonadal sex, ductal sex, and genital sex (17~18). These types of sex forms show obvious differentiation in different periods of each gestational age. Chromosomal sex is determined at the time of fertilization. There is general agreement among investigators that as somites appear in the human embryo, germ cells are identifiable both in the gut and in the mesentery and migrate to the mesonephric ridge by approximately 40 days of gestation, while the extragenital germ cells which remain behind the gut, mesentery or other locations disappear after the eighth week of intrauterine development. Gonadal sex maturation begins at approximately 50 days of gestational age, and simultaneously with development of gonad, genital duct embryogenesis proceeds from either the mesonephric system or the paramesonephric ducts. The development of the female genital ducts and external genitalia does not involve gonadal control. On the other hand, male ductal and external genital differentiation requires androgen secretion by fetal Leydig cells and secretion of Muellerian inhibiting substance of locally influencing hormone by fetal Sertoli cells (17~19). The sexual dimorphism of the human gonad first becomes apparent with the appearance of the seminiferous cords in the fetal testis between 6 and 7 weeks of gestation. The somatic cells of the gonad undergo partial organization into testis as specified by genotypes, even if the germ cells are prevented from migrating to the genital ridge (19).

The hypothesis of an upregulatory gene which controls the sequential differentiation in male sex lies within the Y chromosome has vigorously been raised. It appears that some Y chromosomal material, not the entire Y chromosome, is probably required for testicular differentiation upon which male ductal and external genital development depends (1~4, 6). The prime locus has been laid near the centromere of the Y chromosome, particularly on its short arm, which has been denominated as a testicular determination factor, TDF in human and Tdy in mice. In the past, several genes proposed as candidates for Tdy/TDF have subsequently been dismissed on the grounds of inappropriate location or expression (20~22). Recently, a master regulatory gene for TDF has been described (5~10) as Sry (sex determining region of Y chromosome), Sxr in mice. Within this candidate, 35 kb of pseudoautosomal boundary region, an open reading frame (ORF) was isolated, that is a part of gene termed Sry which encodes factor necessary for the initiation of

testis formation and lies at the pY53.3 (2.1 kb).

Several extensive molecular detection studies with primers and oligonucleotide probes of Sry have been accomplished (5~10). By ISH and PCR study using Sry mRNA, Sry was expressed in one of the somatic cells lineages present only in the developing gonad, not in the germ cells (23~26). Our results which revealed positive signal for Sry, all MPH with mixed gonadal dysgenesis having Y chromosome showed obvious, though variable, positivity inside the seminiferous tubules. All tubules were entirely immature and composed of only Sertoli cells without any germ cells. Thus, positive signals were majorly detected in the Sertoli cells with minor proportion of interstitial cells, but not in a single germ cell. This result is not inconsistent with the previous study employing in situ hybridization, Y chromosome was decondensed only in Sertoli cells of the adult mouse (23~27). Whereas it is still arbitrary that decondensation of the Y chromosome in Sertoli cells coincides with the occurrence of mature sperm nuclei in another study using in situ hybridization with a probe of pY353/B (27). Though we were not able to study in the adult testis with adequate maturation of germ cells, all cells positive for Sry in MPH were Sertoli cells. Testicular appendage including ductuli efference and ductus deference never revealed any positive reaction for Sry probe. The reason all Sertoli cells did not stain for Sry gene seemed to be rather a thin section insufficient to expose whole nucleus. Thus, these kinds of studies have been focused on the prenatal embryo when determining sex (28~29). Minor proportions of studies has been alotted to the differentiation of intersex such as true or pseudo hermaphrodite or rare sex reversal (9~13). Our study was aimed to lighten the implication of Sry in gonadal tissue of several kinds of intersex and practical utility in diagnostic pathology. In our result, only one of 4 TH contained Sry gene, while one XX reversal were positive for it. Previous studies about XX TH and XX sex reversal denotes that they are a genetically heterogeneous condition, some having TDF sequences (12, 13, 30), but others not (12, 31). Our study in which only one has something to do with Sry sequence as testicular formation is consistent with earlier investigators who reported that some Y sequences were absent in patients with 46XX TH. The possibility that an autosomal dominant mutation could lead to testicular development in patients who lack Y material has been reviewed in 46 XY gonadal dysgenesis associated with Wilm's tumor (32) and in Camptomeric dwarfism (33), with studies of transgenic mice (34). Others have insisted that the possible role of X chromosome in testicular development (35~36). The incomplete testicular differentiation in the patients who had Sry can be explained by a variety of mechanisms (11) : 1) rearrangement or mutation in the Sry locus that occurred during translocation ; 2) diminished expression of Sry due to flanking sequences at the site of Y translocation ; 3) absence of Y sequences other than Sry; 4) the time, location or extent of X inactivation involving the translocated X, which has been known for the most probable site of translocation so far. Our result implied partial testicular differentiation when devoid of Sry, resulted from autosomal mutation rather than the role of Sry, while when present of Sry, probably due to an inactivation of translocated X or mutation of Sry locus. By convention, five XX males evaluated by Ferguson-Smith et al. failed to demonstrate any Y sequences including zinc finger Y (ZFY) or Sry (30~31). Thus 46XX subjects without Sry in TH may have a mutation of an autosomal gene that permits testicular determination in spite of the absence of TDF. The only differential point between XX TH and XX sex reversal lies a degree of testicular determination, the former is differentiated incompletely, the latter completely (12).

Fluorescent in situ hybridization (FISH), a powerful new tool for genetic evaluation, permits microscopic identification and localization of aberrations in the context of celllar and tissue morphology within 36 hours (16). The principles that underlie FISH technology are 1) that DNA single strands can bond to form a duplex, 2) that greater similarity (complementarity) between the strands result in tighter bonding, and 3) that specific segments of DNA (genes or chromosomal regions) can be tagged indirectly with biotin or with digoxigenin, reacted with avidin or antibody, and then linked with either an immunoenzymatic or fluorescent label, or directly with immunofluorescent label as well (15~16). Of course, direct labelled-probe may yield weaker signals than indirect labelled probe, the latter one permits amplification of a fluorescence signal. FISH is applicable to fresh cytologic preparations and to aged cells in paraffin sections. Though PCR techniques enable to recognize alteration in fewer cells than does FISH, FISH offers some unanimous advantages among molecular techniques. The maintenance of architectural integrity permits integration of the extent of genetic aberration and localization of signal in direct relation to tumor morphology. Because a section need not be restricted to tumor cells, stromal or representative normal cells of the same tissue type present on the same slide offer internal controls for reagents. FISH appears to be particularly valuable in assessment of chromosomal genetic alteration, qualification of Sry, in tiny fragment of gonadal tissues with preservation and explicite identification of tissue in detail.

In conclusion, Sry gene can function as testicular determination in almost all intersex except in TH con-

ceivably in a different pathogenesis, FISH might be useful to detect the Sry gene, even the translocated Sry gene in case of XX reversal, as well.

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