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Fate of SRY, PABY, DYS1, DYZ3 and DYZ1 loci in Indian patients harbouring sex chromosomal anomalies

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We analysed chromosomes, conducted hormonal assays and screened genomic DNA of 34 patients with or without detectable Y chromosome for the presence/absence of SRY, PABY, DYS1, DYZ3 and DYZ1 loci and for mutations in the *SRY* gene. The samples studied represented cases of oligozoospermia, cryptorchidism, Swyer syndrome, Turner syndrome, male pseudohermaphroditism, XXY female syndrome, Klinefelter's syndrome, repeated abortion and instances of male infertility. Chromosomal constitutions and the level of hormones (FSH, LH, PRL, E2 and TSH) were found to be abnormal in several cases. A phenotypic female (P20) positive for all the Y-linked loci screened, showed mutations upstream of the HMG box in the *SRY* gene. In addition, one or more of the Y-linked loci were detected in several phenotypic females. Fluorescence in-situ hybridization of metaphase chromosomes and interphase nuclei of an aborted fetus with DYZ1 probe detected signals from normal to low levels to its complete absence confirming a complex Y chromosome mosaicism. Upon DNA analysis, the fetus was found to be positive for all the above-mentioned Y-linked loci. Organizational variation within the DYZ1 arrays and its correlation with recurrent spontaneous abortion may be followed-up in subsequent studies to substantiate this observation. This would augment genetic counselling to the affected couples. Prospects of this approach in the overall management of clinical cases with sex chromosome-related anomalies are discussed.

Key words: indels/oligozoospermia/SRY gene/Turner syndrome/Y-linked loci

Introduction

The human Y chromosome is reported to have about 76 protein coding genes, though only 27 distinct proteins have been identified thus far (Page, 2004). Some of these genes are envisaged to be involved in the control and regulation of spermatogenesis (Lahn and Page, 1997; Skaletsky *et al.*, 2003). Earlier thought to be a genetic wasteland, the Y chromosome is now found to have expanded its repertoire during the course of evolution by sequence amplification and selectively importing genes from autosomes and X chromosomes (Saxena *et al.*, 1996; Skaletsky *et al.*, 2003). Contrary to earlier belief, the Y chromosome was found to have gene-rich palindromes of unprecedented magnitude (Kuroda-Kawaguchi *et al.*, 2001; Skaletsky *et al.*, 2003). Despite gene conversion and palindromic arrangement of the sequences maintaining structural integrity of the Y chromosome, organizational and/or numerical changes leading to sex chromosome anomalies are often encountered. These changes include XY gonadal dysgenesis (Swyer syndrome), XYY males, recurrent spontaneous abortions (RSA), Klinefelter's syndrome and Turner syndrome (TS). The patients with XY female type of gonadal dysgenesis (Swyer syndrome) appearing normal at birth fail to develop secondary sexual characters at puberty, have 'streak gonads' and are unable to menstruate. Small deletions in the short arm of the Y chromosome resulting in 46,XY females have been reported (Disteche *et al.*, 1986). The other type of XY female gonadal dysgenesis is also caused due to mutation in the Y chromosome (Berta *et al.*, 1990; Jager *et al.*, 1990). The XYY syndrome, estimated to occur once in a thousand live births is correlated with tall stature, dull intelligence, delayed speech and some learning problems (Fryns *et al.*, 1995). Similarly, in case of TS, with 45,XO chromosome constitution occurring in the range 1:2000 to 1:5000 female live births (Hook and Warburton, 1983), over 90% of the pure XO conceptuses are eliminated during early prenatal development. After birth, chromosomal mosaicism involving both the X and the Y chromosomes have been reported. The commonly encountered TS karyotypes include 45,X; isochromosome X; 45,X/45,XX; 45,X/46,XY; 45,Xi(Xq); 46,Xi(Yp); 46,Xt(X;X); 46,Xi(X;Y); 46,Xi(Xp); 46,X,del(Xq) r(X); 46,Xr(X) (including small ring X chromosome); 45,/X/46,XX; 45,X/47,XXX; 46,X/46,XX/47,XXX; 45,X/46,XY; and 45,X/46,XXq+46,X+mar(X); 47,XXq +, + mar(X); 46,X+mar(Y); 47,XXq+, + mar(Y) (Ogata and Matsuo, 1995). Based on chromosomal imbalance, genomic imprinting and haploinsufficiency, attempts have been made to explain the genetic basis of the TS phenotype (Ogata and Matsuo, 1995) involving loci in the pseudoautosomal regions on the short arms of the X and Y chromosomes (Ogata *et al.*, 1995; Joseph *et al.*, 1996; Schwinger *et al.*, 1996). However, the number and type of genes likely to be responsible for TS phenotype remain unclear. The only TS feature present consistently in patients with small distal Xp deletions is their short stature, suggesting that the loci responsible for other features lie outside the pseudoautosomal region (Spranger *et al.*, 1997). During the process of developing a physical map of the human Y chromosome, a total of 758 DNA markers were identified of which 136 were found to have multiple locations in the non-recombining regions of the Y chromosome (Tilford *et al.*, 2001). Ideally, these

markers may be checked in a clinical setting to uncover their possible involvement with abnormal phenotypes. However, logistic constraints may not allow such analysis during routine screening of the patients' DNA samples. We analysed genomic DNA of 34 patients with or without cytogenetically detectable Y chromosome for the presence/absence of DYZ1, DYS1, DYZ3, PABY and SRY loci encompassing both the arms, and conducted chromosomal analyses and hormonal assay. Several phenotypic females were found to harbour one or more of the Y-linked loci and showed multiple point mutation(s). This work broadens our understanding of the mutational profile of the Y-linked loci, leading to more accurate DNA diagnosis and augmenting genetic counselling to the affected couples.

Materials and methods

Collection of blood samples and genomic DNA isolation

Blood samples were collected with informed consent from normal males, females, patients and donors/volunteers from J.N. Medical College, Aligarh, India, strictly in accordance with the institute's ethical and biosafety committee. Collection of these samples spanned a period of about 9 years. Genomic DNA from a total of 34 patients together with normal males was isolated following standard protocols (Ali *et al.*, 1986). Of these samples, 19 (P2A/2, P2B/14, P2C/16, P3, P4, P5, P6, P7, P8, P9, P10, P11, P15, P65971, P65972, P65975, P6697, P17698, PHK-459) represented TS, three (P18, P20, PR-137) Swyer syndrome, six (P1, P13, P65973, P65974, P7797, P060600) cryptorchidism and hypogonadism, two (P060500MS, P250500) RSA and one each of Klinefelter's syndrome (P19), male pseudohermaphroditism (P12), oligozoospermia (P60199bl) and XXY female syndrome (P21).

Primers and probe for PCR amplification of genomic DNA and hybridization

A set of oligoprimers representing four different loci (PABY, SRY, DYZ3 and DYS1) of the Y chromosome encompassing both the arms were used for PCR amplifications following standard protocols (Bashamboo *et al.*, 2003). For the SRY locus, two sets of primers were selected. RG4 and RG7 primers specific to a 231-base pair band were used for typing all the samples. The other set of primers encompassing the HMG box, which generated a 612 base pair fragment, was used for analysing the DNA from the phenotypic female(s) positive for all the five Y-linked loci. The details of the probe and PCR primers, their annealing temperatures, size of the expected amplicons and corresponding references are given in Table I.

Restriction digestion, agarose gel electrophoresis and Southern blot hybridization

Approximately 0.5 μg of genomic DNA was digested with *Hae* III enzymes in a 20 μl reaction volume, following the supplier's specifications (New England Biolabs, USA). Digested DNA was electrophoresed on a 20 cm long 1.5% agarose gel in 0.5 × Tris/borate/EDTA buffer (pH 8.2). The DNA was transferred onto the nylon membrane (Southern, 1975) and UV fixed using Strata linker (Stratagene, San Diego, USA), and the blots were hybridized with oligoprobe OAT20Y as mentioned earlier (Gauri Bala *et al.*, 1996). The OAT20Y probe uncovered a 3.4 kb band representing the Yqh region of the human Y chromosome.

Chromosome analyses and estimation of hormonal levels

Wherever possible, chromosome analyses of the patients were conducted following standard protocols (Gauri Bala *et al.*, 1996). However, due to logistic constraints, karyotype analyses of several cases could not be done. For assessing hormonal profiles, 100 μl of serum from each sample was used to estimate E2, LH, TSH, PRL and FSH levels by radioimmunoassay using commercial kits purchased from Bhabha Atomic Research Center, Bombay and Immuno Corp., Canada following the supplier's specifications

Fluorescence in-situ hybridization (FISH)

In an independent study, a blood sample from an aborted fetus (not listed in Table II) was used for chromosome preparation and for analysing the presence/absence of the Y chromosome using FISH. Metaphase chromosomes were prepared following standard methods (Gauri Bala *et al.*, 1996). FISH was conducted with a labelled DYZ1 cloned probe using a Nick Translation Kit from Vysis (Illinois, USA). Hybridization, washing, counterstaining and mounting of the slides with DAPI were conducted following established protocols (Rahman *et al.*, 2004). The slides were screened under the Olympus fluorescence microscope (BX 51) fitted with a vertical fluorescence illuminator U-LH100HG UV, excitation and barrier filters. Metaphase images were captured with a CCD camera and chromosomes were karyotyped using Cytovision 2.81 software from Applied Imaging Systems.

Mutational analysis of the SRY *gene*

Mutational status of the *SRY* gene in patients was assessed by PCR, followed by cloning and sequencing of the resultant amplicons. In order to ascertain the authenticity of the mutations detected in other phenotypic females, the *SRY* sequences from four normal males, amplified by PCR, were cloned and sequenced. In addition, PCR products were also used for direct sequencing. The *SRY* sequences from four normal males (accession nos. AY601852, AY601853, AY601854 and AY601855) and five phenotypic female patients (accession nos. AY601856, AY601857, AY601858, AY601859 and AY601860) were deposited with GenBank. The patients' DNA sequences were subjected to multiple alignments with normal DNA (accession no. X53772) downloaded from GenBank using the CLUSTALW program on the default server (http://www.ebi.ac.uk/clustalw).

Results

Y chromosome variability in patients with genetic anomalies

Hormonal and chromosomal profiles of the patients studied were found to be abnormal in several cases compared to that of

Table I. Y chromosome-related PCR primers and hybridization probes used for assessing different loci

S. no	Loci	Set of primers	Annealing temp. (°C)	Amplicon size (bp)	Reference
1	SRY, RG4 and RG7	(i) 5′ GGTCAAGCGACCCATGAAYGCNTT 3′ (ii) 5′ GGTCGATACTTATAGTTCGGGTAYTT 3′	55	231	Griffiths and Tiwari, 1993
2	SRY2 and SRY3	(i) 5′ CCCGAATTCGACAATGCAATCATATGCTTCTGC 3′ (ii) 5′ CTGTAGCGGTCCCGT TGCTGCGGTG 3′	65	612	Nagafuchi *et al.*, 1992
3	PABY	(i) 5′GTACTACCTTTAGAAAACTAGTATTTTCCC 3′ (ii) 5′GAATTCTTAACAGGACCCATTTAGGATTAA 3′	54	970	Nagafuchi *et al.*, 1992
4	DYZ3	(i) 5′ATGATAGAAACGGAAATATG 3′ (ii) 5′AGTAGAATGCAAAGGGCTCC 3′	54	120	Nagafuchi *et al.*, 1992
5	DYS1	(i) 5′ AATAGAGCCTTATCAGCAGA 3′ (ii) 5′ AGTCAGTCTGGATGTTTCAG 3′	54	120	Nagafuchi *et al.*, 1992
6	DYZ1	5′ TTCCATTCCATTCCATTCCA 3′ (OAT20Y oligo probe)	Southern hybridization		Gauri Bala *et al.*, 1996

Note: (i) and (ii) represent forward (5′) and reverse (3′) primer sequences, respectively. For each reaction, reproducibility of the results was confirmed by conducting PCR amplification on three occasions.

Table II. Details of the clinical, chromosomal and hormonal profiles of patients

S. no	Patient	Phenotype sex	Karyotype	Clinical features	Anomaly status/syndrome	Hormonal profile	SRY	PABY	DYZ3	DYS1	DYZ1
1	NM	M	46,XY	Normal[a]	Normal	FSH-5–20 IU/l LH-0.007–0.024 IU/l	+	+	+	+	+
2	NF	F	46,XX	Normal[a]	Normal	FSH-3-20 IU/l LH-5–20 IU/l PRL-10–25 μg/l	−	−	−	−	−
3	P1	M	46,XY	Hypogonadism, undescended testis	Cryptorchidism and hypogonadism	FSH-13.2 IU/l LH-27.0 IU/l	+	+	+	+	+
4	P2A/2	F	46XX/46XY/47XXXp-	Primary amenorrhoea, SSC absent, hypogonadism, uterus not seen, external genitalia normal, no skeletal deformation, webbing of neck	Turner syndrome	LH-42.0 IU/l FSH-46.0 IU/l PRL-24.5 μg/l TSH-3.2 nm/l	+	+	+	+	+
5	P2B/14	F	45,XO 46,XX 46,XY	Primary amenorrhoea, no breast or pubic hair development, USG shows small, nodule type hypoplastic uterus	Turner syndrome	LH-37.0 IU/l, FSH-450.0 IU/l, PRL-27.0 μg/l	+	+	+	+	+
6	P2C/16	F	NA	Primary amenorrhoea, underdeveloped breasts, USG shows an underdeveloped and small uterus	Turner syndrome	FSH-72.0 IU/l PRL-14.0 IU/l	+	+	+	−	−
7	P3	F	NA	Short stature, webbed neck, primary amenorrhoea, breast and external genitalia normal, normal pubic and auxiliary hair	Turner syndrome	FSH-3.0 IU/l E2-54.0 pg/ml PRL-7.0 μg/l	−	−	−	−	−
8	P4	F	45,XO 46,XX 46,XY	Turner variant, Primary amenorrhoea, USG reveals extremely hypoplastic uterus, breast and external genitalia were underdeveloped, Sparse auxiliary pubic hair	Turner syndrome	LH-27.0 IU/l, FSH-40.0 IU/l, PRL-22.0 μg/l	+	+	+	+	+
9	P5	F	45,XO/46,XX	Primary amenorrhoea, USG shows no evidence of a uterus or ovaries, underdeveloped breast, sparse pubic hair	Turner syndrome	LH-56.0 IU/l FSH-48.0 IU/l PRL-105.0 μg/l	−	−	−	−	−
10	P6	F	46,XX/47,XXXp-	Short stature, webbed neck, primary amenorrhoea, USG shows hypoplastic uterus and ovaries, normal external genitalia	Turner syndrome	LH-22.6 IU/l FSH-7.0 IU/l	−	−	−	−	−
11	P7	F	45,XO/46,XX/47,XXX	Short stature, webbed neck, primary amenorrhoea, USG reveals small uterus, atopic external genitalia	Turner syndrome	LH-53.0 IU/l FSH-47.0 IU/l	−	−	−	−	−
12	P8	F	46,XX/47,XXX	Short stature, primary amenorrhoea, poorly developed SSC	Turner syndrome	NA	−	−	−	−	−
13	P9	F	46,XX/47,XXX	Short stature, primary amenorrhoea, poorly developed SSC, USG does not reveal a uterus, dysgenic gonads, abdominal testis were surgically removed	Turner syndrome	NA	−	−	+	+	+
14	P10	F	45,XO/46,XX	Short stature, webbed neck, shield-like chest, secondary amenorrhoea	Turner syndrome	NA	−	−	−	−	−
15	P11	F	45,XO 46,XX 47,XX, idic (Yq)	Short stature, shield-like chest, no breast nodules, auxillary hair or pubic hair, primary amenorrhoea, absence of SSC, external genitalia normal, USG did not show an ovary but a streak gonad was observed	Turner syndrome	NA	−	−	+	+	+
16	P12	M	NA	Male pseudohermaphroditism, external genitalia ambiguous	Male pseudohermaphroditism	NA	+	+	+	+	+
17	P13	M	46,XY	Cryptorchidism, USG did not show testis in inguinal and abdominal region	Cryptorchidism and hypogonadism	NA	+	+	+	+	+
18	P15	F	45,XO/46,XX	Primary amenorrhoea, atopic vagina, small uterus seen during gynaecological examination, no endometrial tissue seen in biopsy, USG showed anteverted and anteflexed uterus	Turner syndrome	FSH-0.075 IU/l LH-0.062 IU/l PRL-6.4 μg/l	−	−	−	−	−

Table II. *Continued*

S. no	Patient	Phenotype sex	Karyotype	Clinical features	Anomaly status/syndrome	Hormonal profile	SRY	PABY	DYZ3	DYS1	DYZ1
19	P18	F	46,XY	Physically resembles a normal female, with normal external genitalia	Swyer syndrome	NA	−	+	+	+	+
20	P19	M	46,XX	Klinefelter's syndrome, external genitalia and testis are apparently normal, well-developed breasts		NA	+	+	+	+	+
21	P20	F	46,XY	A phenotypic female with male internal organs, absence of uterus	Swyer syndrome	NA	+	+	+	+	+
22	P21	F	47,XXY	Ambiguous genitalia, more like a male, no vaginal opening, USG showed ovaries and uterus is smaller in size. No testis or testicular tissue seen		NA	+	+	+	+	+
23	P65971	F	46,XX/46,XY/ 47,XXXp-	Turner variant, primary amenorrhoea, tall stature	Turner syndrome	NA	+	+	+	+	+
24	P65972	F	NA	Primary amenorrhoea	Turner syndrome	NA	−	+	+	+	−
25	P65973	M	NA	Cryptorchidism and hypogonadism	Cryptorchidism and hypogonadism	NA	+	+	+	+	+
26	P65974	M	NA	Cryptorchidism and hypogonadism	Cryptorchidism and hypogonadism	NA	+	+	+	+	−
27	P65975	F	NA	Turner syndrome	Turner syndrome	NA	−	−	−	−	−
28	P6697	F	NA	Short stature, hirsutism, primary amenorrhoea	Turner syndrome	NA	+	+	+	−	−
29	P7797	M	NA	Cryptorchidism	Cryptorchidism and hypogonadism	NA	−	+	+	+	+
30	P17698	F	NA	Short stature, shield-like chest, primary amenorrhoea, USG revealed hypertrophied kidneys, kidney showed dilated pelvis and calyces, ureter not seen, uterus not seen, vaginal echo present, ovaries absent	Turner syndrome	FSH-0.012 IU/l LH-0.032 IU/l, PRL-8.5 μg/l	+	+	+	+	+
31	PHK-459	F	45,XO/46,X(mar)	Primary amenorrhoea, normal developmental milestones, proportionate but short stature, sparse auxiliary and pubic hair, normal external genitalia, no webbing of neck, no breast nodules seen. USG revealed both kidneys normal in size, shape, outline and echotexture. Uterus is small and infantile. Ovaries not seen	Turner syndrome	E2-0.01 pg/ml FSH-52.33 IU/l LH-23.37 IU/l	−	−	+	+	−
32	P60199bl	M	NA	Infertile male, oligospermic	Oligozoospermia	FSH-4.2 U/l, LH-2.1 IU/l, PRL-14.0 μg/l, TSH-7.5 ng/ml	+	+	+	+	+
33	P060600	M	NA	Hypogonadism	Cryptorchidism and hypogonadism	NA	+	+	+	+	+
34	P060500MS	F	NA	Recurrent spontaneous abortions	Recurrent spontaneous abortions	NA	−	+	−	−	−
35	P250500MS	F	NA	Recurrent spontaneous abortions	Recurrent spontaneous abortions	NA	−	−	+	−	−
36	PR-137	F	46,XY	Swyer syndrome	Swyer syndrome	NA	+	+	+	+	+

[a]Normal ranges of hormones in males and females are given. M = male; F = female; NA = not available; SSC = secondary sexual characters; USG = ultrasonography; PRL = prolactin releasing hormone; LH = luteinizing hormone; FSH = follicle stimulating hormone; TSH = thyroid stimulating hormone.

the normal ones (Table II). PCR-based analysis and Southern blot hybridization showed presence/absence of the Y-linked loci in several cases where the Y chromosome was not detectable cytogenetically. Of the 19 cases of TS analysed, 7 were found to be positive for SRY, 8 for PABY, 11 for DYZ3, 9 for DYS1 and 7 for DYZ1 loci. Interestingly, of all the cases of TS, only 5 samples were positive for all 5 loci, whereas, 7 were found to be negative. Thus, the majority of the patients with TS showed varying levels of aberrant Y chromosome. One sample (P60199bl) representing oligozoospermia was found to be positive for all the 5 loci. The number of patients belonging to different categories of genetic anomalies/syndrome and positive/negative status of different Y-linked loci therein are given in Table III. The amplicons from the representative patient samples corresponding to the SRY, PABY, DYZ3 and DYS1 loci are shown in

Table III. Status of Y-linked loci in patients with sex chromosome-related anomalies

S. no	Clinical features	No of patients	Positivity for different Y-linked loci				
			PABY	SRY	DYZ3	DYS1	DYZ1
1	Turner syndrome	19	8	7	11	9	7
2	Swyer syndrome	3	3	2	3	3	3
3	Cryptorchidism/hypogonadism	6	6	5	6	6	5
4	Repeated spontaneous abortion	2	1	0	1	0	0
5	Klinefelter's syndrome	1	1	1	1	1	1
6	Male pseudohermaphroditism	1	1	1	0	1	1
7	XXY (female) syndrome	1	1	1	1	1	1
8	Oligozoospermia	1	1	1	1	1	1

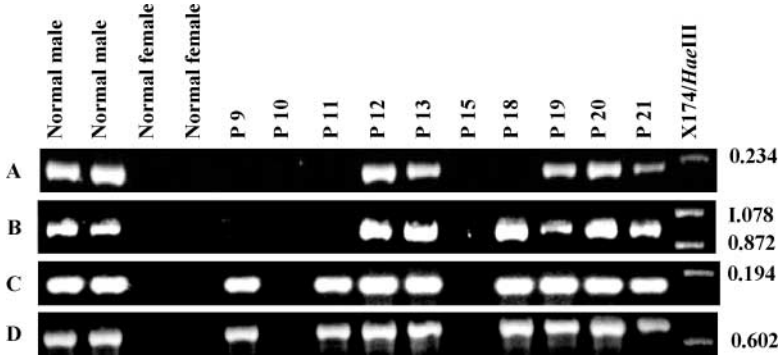

Figure 1. A representative gel showing PCR amplification of patients' genomic DNA. Panels **A**, **B**, **C** and **D** represent *SRY*, *PABY*, *DYZ3* and *DYS1* specific amplicons of 231, 970, 120 and 710 base pairs, respectively. As given in Table I, SRY primers RG4 and RG7 generating a 231 base-pair band were used for screening all the samples. The other set of primers, specific for SRY encompassing HMG box were used for the amplification of genomic DNA from the phenotypic female patients (data not shown). DNA from normal individuals of both the sexes was used as control and ΦX174/*Hae* III DNA (M) as molecular size marker. Of the 34 patients whose DNA were analysed, results of only 10 samples are shown here. The remaining ones have been included in the diagrammatic illustration of Figure 7.

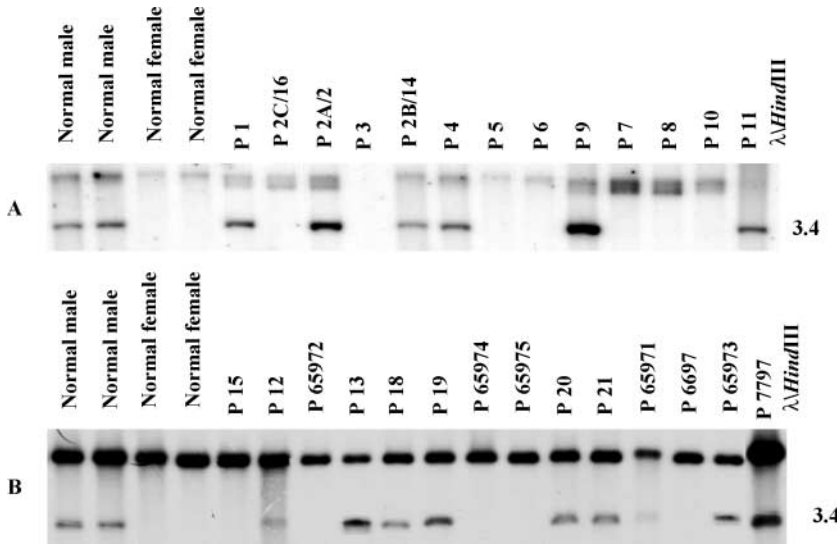

Figure 2. Typing of *Hae* III digested genomic DNA of 27 patients with OAT20Y probe representing DYZ1 locus that shows a 3.4 kb band in the normal males. The remaining seven samples analysed independently, not shown here, have been included in the diagrammatic illustration of Figure 7. Note the presence of a male-specific 3.4 kb band in the patients. λ\HindIII DNA was used as the molecular size marker given in kb.

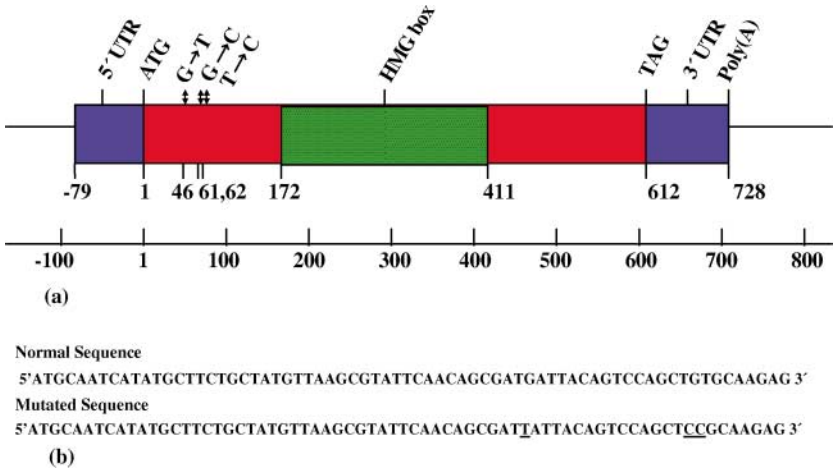

Figure 3. Schematic representation of the novel mutations detected in the *SRY* gene in a patient (P20). Positions of the mutations are marked with arrows in the 5′ non-HMG box region (**a**: upper panel). The lower panel (**b**), shows the sequences (partial) from a normal and a mutant *SRY* gene. Mutations in the patient at nucleotide 46, 61 and 62 are underlined.

Figure 1. The patients' samples positive for the DYZ1, based on Southern blot hybridization, are shown in Figure 2. Of those patients belonging to other categories, one phenotypic male (P7797) suffering from cryptorchidism was found to be negative for the *SRY* sequence. On the other hand, five phenotypic female patients (P2A/2, P21, P65971, P6697 and P20) were positive for the *SRY* gene.

Of these patients P20, an XY female with Swyer syndrome, was positive for all the Y-linked loci. Sequence analysis of the *SRY* gene from this patient (accession no. AY601858), encompassing HMG box, showed transition and transversion mutations (Figure 3). These mutations are predicted to alter the spatial organization of the SRY protein (Figure 4) and may affect its function. In addition, CLUSTALW multiple sequence alignment (www.ebi.ac.uk/clustalw) showed mutations in the 3′ region at nucleotide positions 507 and 564, in patient P20, where G was changed to C and A to T, respectively. However, these mutations did not alter the amino acid sequences. Besides these changes, two deletions were detected at nucleotide positions 567 and 577 involving C at both positions. In a phenotypic female patient, P2A/2, multiple sequence alignment with CLUSTALW showed point mutation in the extreme 5′ region of the *SRY* gene at position 1 changing nucleotide A to T in the start codon. This caused a change in the amino acid from methionine to leucine. Thus, of the five phenotypic females, three (P21, P65971 and P6697) showed normal *SRY* sequences, whereas two (P2A/2 and P20) showed mutations in the *SRY* gene. The result of CLUSTALW alignment of the *SRY* sequences from these patients and normal individuals is given in Figure 5.

FISH analysis of an aborted fetus in an independent study (not listed in the Table II) showed complete absence of DYZ1 signal in some cells and varying levels of this signal in others (Figure 6a) portraying a gross level of Y chromosome mosaicism. Several metaphases from the normal males used as control showed discernible DYZ1-specific signals in the Yqh region of the Y chromosome(s) (Figure 6b). The DYZ1 signals on the long arm of the Y chromosome in normal males were clearly noticeable within the expected range (Figure 6b, C-I) compared to those in the cells devoid of such a signal from the aborted fetus (see Figure 6a). Thus, the present study establishes a correlation between the varying levels of the Y chromosome mosaicism uncovered by the presence/absence of the DYZ1 specific signals and RSA.

Of all the samples analysed (Table II), the patients positive for the PABY locus were 64.7%; the SRY, 52.9%; the DYZ3, 73.5%; the DYS1, 64.7%; and for the DYZ1, 55.9%. However, in cases of TS, the patients positive for PABY were 42.1%; SRY, 36.84%; DYZ3, 57.9%; DYS1, 47.4%; and DYZ1, 36.8%. Interestingly, in both the categories, maximum numbers of samples were found to be positive for the DYZ3 locus. Presence/absence of the Y chromosome-linked loci in patients with their corresponding phenotypic sex is shown in a schematic illustration (Figure 7). The filled blocks correspond to the presence of the loci, whereas the empty ones denote their absence.

Discussion

This study was undertaken with a view to ascertain loss or gain of five well-defined Y chromosome-linked loci in patients suffering from sex chromosome-related anomalies. Several phenotypic female patients were found to be positive for one or more of the Y-linked

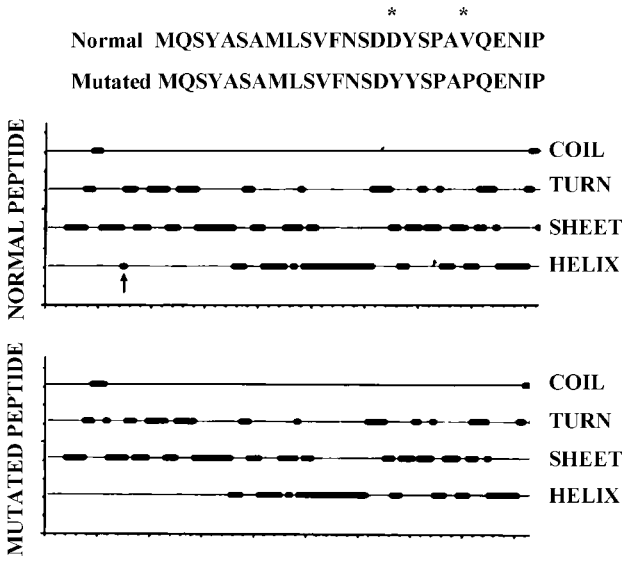

Figure 4. Predicted secondary structure of the SRY peptide encompassing the mutated region using default server (http://dot.imgen.bcm.tmc.edu:9331/seq-search/struc-predict.html). Normal peptide sequences (partial) and mutated ones in the 5′ non-HMG box region at codons 16 and 21 are marked by an asterisk (*). An arrow shows a small helix in the normal SRY peptide, which is absent in the mutated one.

loci. This suggests that Y chromosome mosaicism is not uncommon, though the cause or the effect of such aberration remains unclear. Patients with genetic anomalies showed an abnormal hormonal profile indicating possible involvement of the autosomal genes in the diseased phenotype. This opens up newer vistas for the in-depth analysis of the candidate genes related to paracrine systems.

Mutations in the SRY *gene*

Mutations in the *SRY* gene have been reported in about 20% of the cases with XY gonadal dysgenesis. In the present study, mutations causing substitution of amino acids at codons 16 (Asp → Tyr) and 21 (Val → Pro) in the *SRY* gene result in the loss of a small helix prior to the HMG box in the 5′ region (Figure 4). This altered conformation may affect the spatial organization of the SRY protein, rendering it incapable of functioning accurately as a transcription factor, as demonstrated by the predicted secondary structure analysis. Mutations in the *SRY* gene in Swyer syndrome have been reported to be involved in the sex reversal of the patient. Subject P20 appears to belong to this category substantiating that mutation(s) in the *SRY* gene could be a prime cause for sex reversal.

A phenotypic male patient (P7797) with cryptorchidism and hypogonadism positive for the four loci (PABY, DYZ3, DYS1 and DYZ1) was found to be negative for the *SRY* gene. The resultant phenotype could either be caused by the apparent deletion of the *SRY* gene or mutation(s) in some other genes. The patient (P19) suffering from male pseudohermaphroditism (positive for all the Y loci studied), seemed to have a structurally intact Y chromosome as

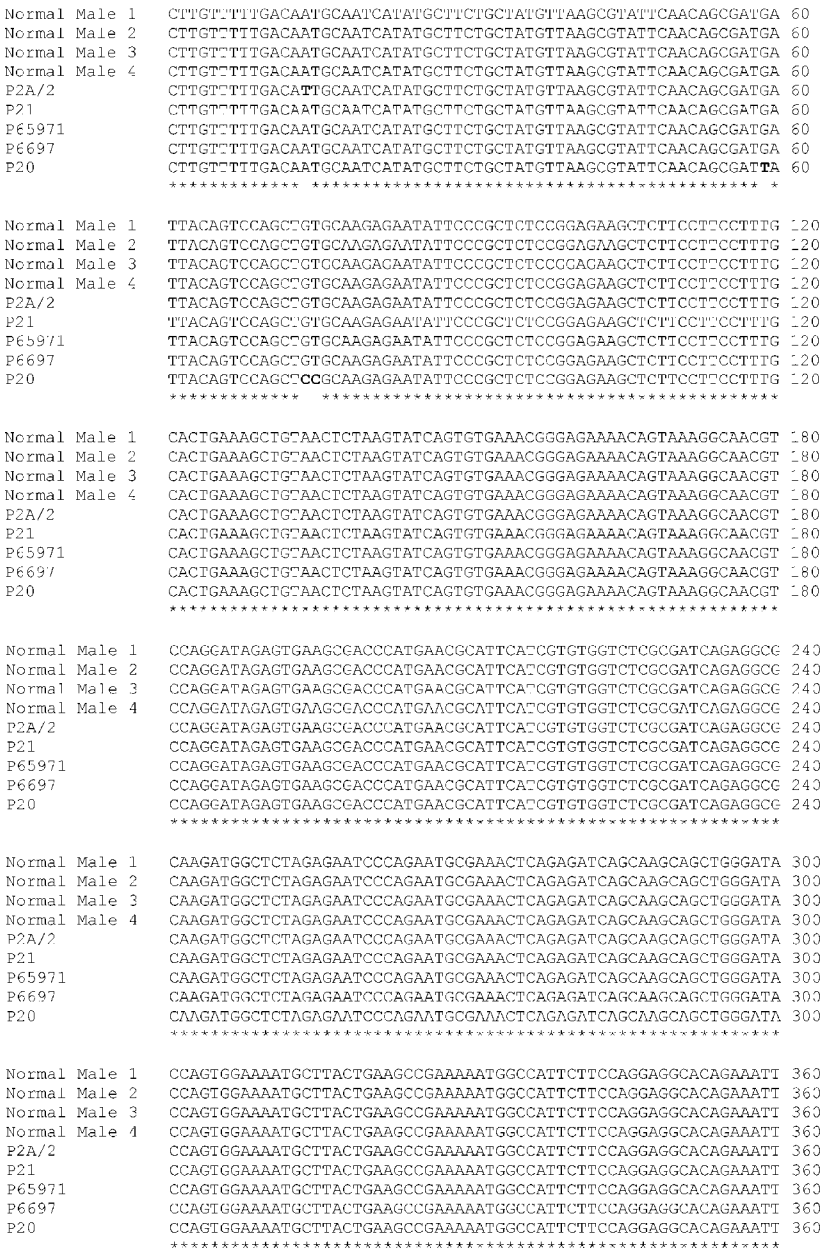

Figure 5. CLUSTALW (1.81) multiple alignment of subset sequences of the *SRY* gene from four normal males and five phenotypic female patients. Note the normal *SRY* sequence in all the males and indels in P20. The patient P2A/2 showed point mutation at the 5′ position of the *SRY* gene resulting in a change in the start codon from methionine to leucine.

Figure 5. (*Continued.*)

revealed by DNA analysis, but the same was undetectable cytogenetically. Like other instances, this could also be a case of acute mosaicism with only fewer cells having a Y chromosome that escaped detection during cytogenetic analysis. A phenotypically male patient (P60199) suffering from oligozoospermia was also found to be positive for all the five loci, indicating the presence of an intact Y chromosome. However, on the basis of screening of a single sample, it is difficult to establish a correlation between presence/absence of these loci and oligozoospermia. In addition, mutation(s) in autosomal genes responsible for such an anomaly may not be ruled out. In a phenotypic female patient with Swyer syndrome (PRK137) and another with XXY syndrome (P21), the *SRY* gene was apparently intact since no mutation was detected. Nonetheless, mutation(s) in the flanking regions of the *SRY* gene leading to its ectopic expression or silencing of this gene may not be ruled out as reported earlier (Kwok *et al.*, 1996; Poulat *et al.*, 1997; Vietia *et al.*, 1997). A phenotypic female proband (P060500MSF) suffering from RSA (Figure 7) was found to be positive for the PABY locus. In the absence of other causes, it is tempting to construe that this region may be responsible for abnormal exchange during zygotic cell divisions, leading to mitotic instability and eventually the loss of fetus. However, in the absence of direct evidence, no such conclusion can be drawn. These observations at best provide indirect evidence that in phenotypic females, presence of one or more Y-linked loci may be the cause of genetic anomalies, though involvement of autosomal genes may not be ruled out.

Y chromosome mosaicism and Turner syndrome

Turner patients with Y chromosomes have 15–20% increased risk of developing gonadoblastoma (Verp and Simpson, 1987) but the actual percentage of Y chromosome-bearing cells in the neoplastic tissue remains unclear. Thus, the assessment of the extent of the Y chromosome mosaicism in the infertile or subfertile cases is of relevance. In this context, we used DYZ1 repeat fraction as probe for FISH and its core sequences for DNA typing. The high copy number of DYZ1 (2000–4000 copies per Y chromosome) facilitated the detection of the Y chromosome even in the aberrant forms as shown in our FISH study (Figure 6a). Cryptic mosaicism of the Y chromosome has been reported to be a rare event and may be less than 1% (Medlej *et al.*, 1992; Larsen *et al.*, 1995; Jacobs *et al.*, 1997; Yorifugi *et al.*, 1997). In our study, Y chromosome mosaicism in Turner cases was found to be about 57%, which is apparently much higher than those reported from US and European populations. In the absence of sufficient data from other parts of India, no conclusion

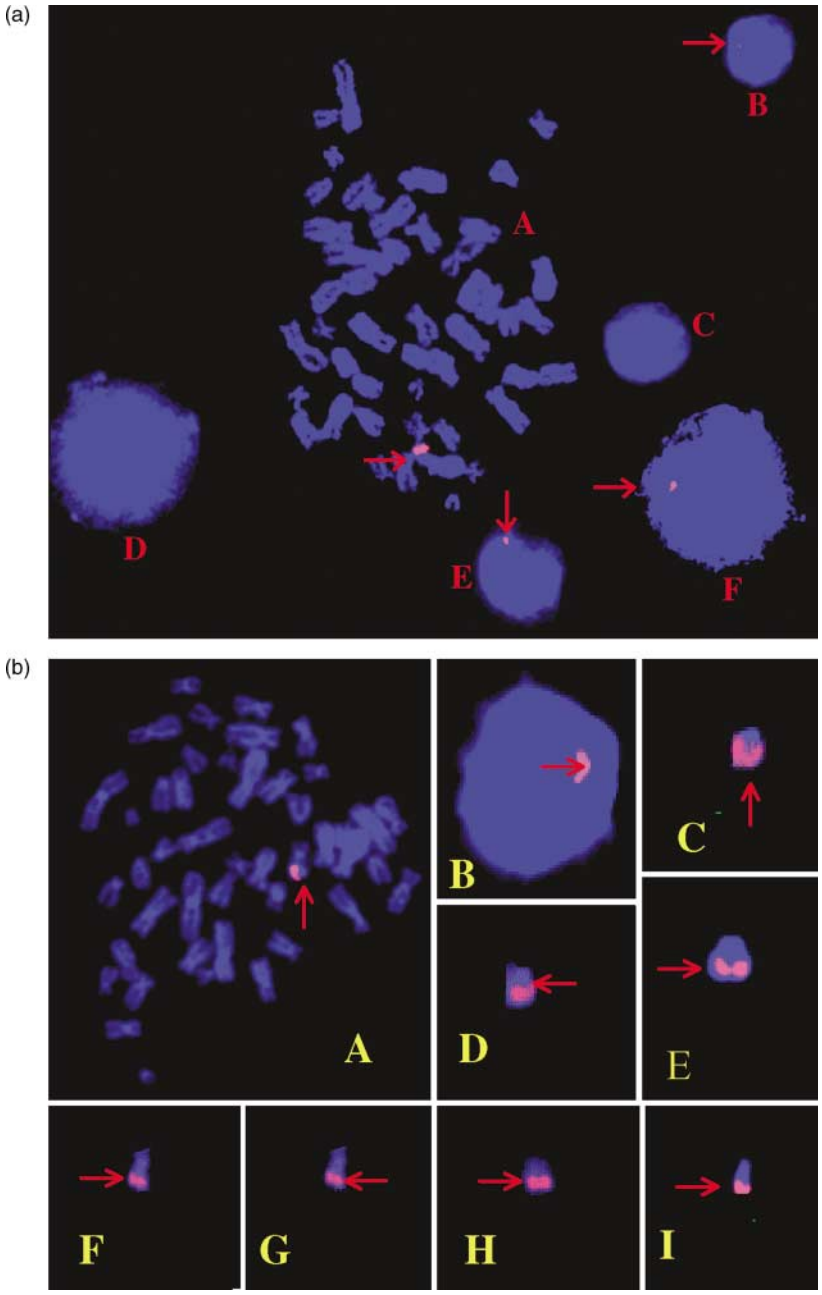

Figure 6a. FISH using DYZ1 probe with metaphase chromosomes (**A**) and interphase nuclei (**B**, **C**, **D**, **E** and **F**) from the blood sample of an aborted fetus (not listed in Table II). **A** shows signal in the qh region of the Y chromosome; **B**, miniscule signal; **C** and **D**, complete absence of signal and **E** and **F**, detectable but reduced levels of signal compared to that of the normal Y chromosome.
Figure 6b. A comparative profile of DYZ1 signals detected by FISH in metaphase chromosomes (**A**) and interphase nuclei (**B**) of the aborted fetus and Y chromosomes from several unrelated normal males (**C–I**). Note the signal variation within the Y chromosomes amongst different individuals.

can be drawn. However, these observations are envisaged to be of great relevance in the context of future studies along these lines.

DYZ1 locus and its (in)stability

A putative role of DYZ1-related sequences for the development of normal males has been postulated (Lau and Schonberg, 1984; Manz *et al.*, 1992). Typing of the patients' genomic DNA with a DYZ1 probe showed an allelic profile similar to that of normal males in more than 50% of cases (Figure 2). However, this typing system does not demonstrate loss or gain of the DYZ1 arrays resulting in copy number variation. FISH uncovers this copy number variation as demonstrated in a sample of RSA in the present study (Figure 6a). We hypothesize that a critical number of DYZ1 copies on the long arm of the human Y chromosome may be necessary for the maintenance of its structural and functional integrity. It has been argued that individuals with loss of almost all (or all) of the heterochromatin have normal male phenotypes. While this may be true, not all such males may be genetically fertile since the deletion of heterochromatin from the Yqh region, at times, may include neighbouring functional gene(s) as well. Analysis of such males for copy number variation of the DYZ1 fraction and assessment of their fertility status would prove to be informative. In the present study (see FISH data in Figure 6a and b), fewer copies of DYZ1 arrays in

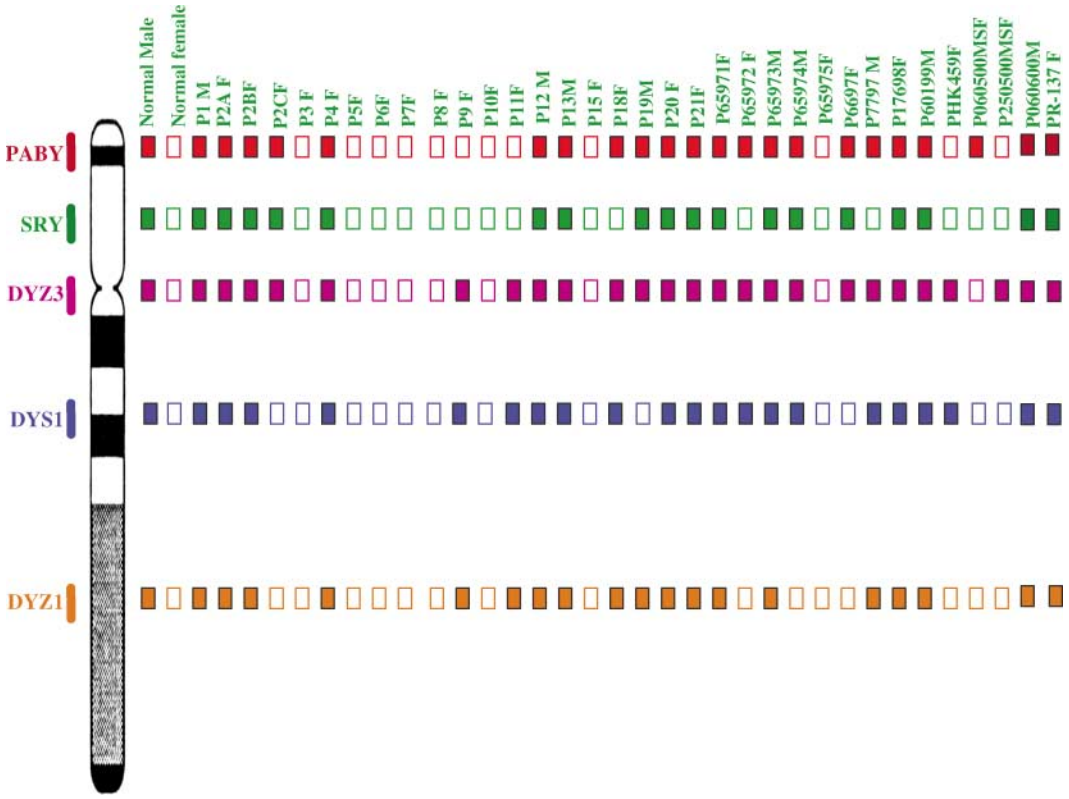

Figure 7. Diagrammatic illustration highlighting the screening results of the patients' DNA with five Y chromosome-linked loci (PABY, SRY, DYZ3, DYS1 and DYZ1) used as markers. The filled blocks denote presence of the loci, whereas the empty ones correspond to absence of the same loci. Several phenotypic females, positive for one or more of these markers, suggest that low levels of Y chromosome mosaicism are common in the patients with sex chromosome anomalies.

some cells and near normal ones in others from RSA may be a true reflection of its still uncharacterized biological significance.

Concluding remarks

Our work shows high levels of Y chromosome mosaicism in Turner cases. For the first time, a wide spectrum of Y chromosome aberrations, encompassing partial loss of DYZ1 fraction to its complete absence was noticed. Further, in several phenotypic females, presence or absence of one or more of the Y-linked loci were observed. Of all the Y-linked loci studied, DYZ3 was found to be present in the maximum number of cases, suggesting its more stable nature and important biological roles. In addition, acute organizational variation within the DYZ1 arrays is correlated with RSA. Irrespective of its biological significance, DYZ1 repeat fraction may be used as a probe to address a number of biological, clinical and forensic issues.

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