Article

Y chromosome mosaicism and occurrence of gonadoblastoma in cases of Turner syndrome and amenorrhoea

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Abstract

In the present study, 73 cases with a clinical diagnosis of Turner syndrome, or with primary or secondary amenorrhoea without frank Turner phenotype, were evaluated for presence of low level Y chromosome mosaicism using molecular methods. Fluorescence in-situ hybridization for centromere and q arm of the Y chromosome and nested polymerase chain reaction for the sex determining region on Y (SRY) gene were performed in peripheral blood, buccal cells and gonadal biopsies. The overall frequency of Y chromosome mosaicism was found to be 18% (13/73 cases). Four cases (16%) of Turner syndrome had Y chromosome mosaicism, seven cases (28%) with primary amenorrhoea and two cases (9%) with secondary amenorrhoea had Y chromosome mosaicism. Histologically detectable gonadoblastoma was observed in one of seven cases (14%) that had Y chromosome mosaicism. This frequency is lower than that reported previously, underscoring the need for large prospective investigations to determine the frequency of Y chromosome mosaicism and occurrence of gonadoblastoma in cases of Turner syndrome and other forms of amenorrhoea.

Keywords: *amenorrhoea, FISH, gonadoblastoma, mosaicism, Turner syndrome, Y chromosome*

Introduction

Three independent roles of the Y chromosome have been identified to date. First, its role in sex determination; second, in regulation of spermatogenesis; and third, in development of gonadal cancers. The association of the Y chromosome and gonadoblastoma has been well established. Several studies have shown that patients with gonadal dysgenesis are at high risk of developing tumours, especially of germ cells and sex cord elements in the presence of the Y chromosome (Manuel *et al*., 1976; Verp and Simpson, 1987; Canto *et al*., 2004; Bianco *et al*., 2006; Brant *et al*., 2006).

Amongst the patients at risk for developing gonadoblastoma are those with pure gonadal dysgenesis, testicular feminization and true hermaphrodites who carry a Y chromosome in their genome. Studies have estimated a risk of ~15–20% (Schellhas, 1974), which seems to increase up to 30% by the age of 30 years (Manuel *et al*., 1976; Verp and Simpson, 1987). As the tumour commonly progresses into invasive dysgerminoma, early surgical removal of the gonads is recommended for patients at risk of developing gonadoblastoma.

Individuals with Turner syndrome (45,X) and its variants also have gonadal dysgenesis but are not at risk of developing gonadoblastoma as they do not have a Y chromosome or its fragment. However, accurate detection of the Y chromosome or its fragment is clinically important in this class of patients as the combination would predispose the patients to the malignancy. Indeed, recent studies have reported that almost 40% of Turner syndrome patients that carry sequences of Y chromosome in their genome develop gonadoblastoma (Canto *et al*., 2004; Brant

et al., 2006). However, the frequency of occurrence of the Y chromosome (translocation or more commonly mosaicism) in Turner syndrome patients is controversial. Using conventional cytogenetic analysis, the percentage of mosaicism with a cell line containing a normal or abnormal Y is estimated to be approximately 5% (Magenis *et al*., 1980; Suri *et al*., 1995; Gravholt *et al*., 2000); half the number of marker chromosomes found in Turner syndrome patients (at a frequency of 3%) are Y derived (Magenis *et al*., 1980; Lindgren *et al*., 1992). In contrast, using sensitive molecular methods, the frequency of Y chromosome mosaicism in Turner syndrome is reported to be highly variable (Ostrer and Clayton, 1989; Medlej *et al*., 1992; Kocova *et al*., 1993; Binder *et al*., 1995; Pastalis *et al*., 1998; Alvarez-Nava *et al*. 2003). Owing to the discrepancies between different studies, no consensus exists regarding the necessity to screen Turner syndrome cases for the presence of the Y chromosome using molecular methods.

Another group of patients that are at risk of developing gonadoblastoma are cases with primary or secondary amenorrhoea without frank features of Turner syndrome. While the causes of amenorrhoea in many cases are multiple, gonadal dysgenesis along with Y chromosome (which might exist in some of these cases) may predispose those patients towards developing the cancer. However, no information is available on the frequency of Y chromosome mosaicism in cases with primary or secondary amenorrhoea without features of Turner syndrome.

The purpose of this study was to determine the incidence of low level Y chromosome mosaicism in cases of Turner syndrome and amenorrhoea using molecular methods, and to determine the occurrence of gonadoblastoma in the Y-chromosome-positive cases.

Materials and methods

Patients

A total of 73 cases with clinical suspicion of Turner Syndrome or amenorrhoea (primary or secondary; **Table 1**) referred to the Genetic Clinic of BJ Wadia Hospital (a secondary referral centre) that met the following inclusion criteria were selected for cytogenetic analysis. The inclusion criteria for Turner syndrome were female phenotype, short stature, lack of pubertal maturation and any of the associated anomalies (Saenger *et al*., 2001). The cases with no menstrual history and absence of typical Turner stigmata were classified as having primary amenorrhoea. Cases with cessation of menstruation (>6 months) in the absence of pregnancy or before the age of menopause were grouped as having secondary amenorrhoea. Cases diagnosed with androgen insensitivity syndrome, prolactinaemia or other endocrine disorders were excluded. In none of the cases did the ovaries appeared enlarged on pelvic ultrasound examination.

Table 1. Details of the patients included in this study.

Clinical diagnosis	*No. of cases*	*Age range*
Turner syndrome	25	6 days–17 years
Primary amenorrhoea	25	16–23 years
Secondary amenorrhoea	23	22–29 years

Sample collection and processing

Peripheral blood was collected in heparinized syringes from all the cases. In 40 cases, buccal cells were scraped from the inner lining of the cheeks and suspended in saline. Gonadal tissue sample was obtained after gonadectomy or diagnostic biopsy in 11 cases.

Mononuclear cells were isolated from blood and processed according to standard protocol. Buccal cells were washed three times in saline and processed as described previously (Modi *et al*., 2003a). Gonadal samples were fixed overnight in 10% buffered formalin and processed for routine paraffin embedding and sectioning.

Karyotyping

Karyotyping was performed using standard 72 h cultures and the metaphase spreads were stained using the G banding technique. A total of 15–20 metaphases per case were counted and two were karyotyped using the standard criteria.

Fluorescent in-situ hybridization

The sex chromosome genotype (X and Y chromosome status) of the patients was examined by fluorescent in-situ hybridization (FISH) using dual X- and Y-chromosome-specific probes (Vysis, UK). Two separate sets of XY-chromosome-specific probes were used in this study. While the X chromosome probe detected the centromere in both the sets, the Y chromosome probe detected the centromeric sequence in one set, and the other FISH probe set spanned the region complementary to satellite III on the q arm of the Y chromosome. All patients were screened using both probes.

FISH was performed on mononuclear cells and buccal cells according to the protocol detailed previously (Modi *et al*., 1999, 2003a). Between three and five paraffin tissue sections (20 μm thick) were dewaxed, hydrated, and cells were isolated and used for FISH according to the protocol detailed earlier (Modi *et al*., 2003b). The slides were viewed under a fluorescence microscope (Olympus BX-60, Japan) using a triple-band pass filter (Vysis). At least 500 mononuclear cells or 200 buccal or gonadal cells were scored for the number of signals according to the criteria described previously (Modi *et al*., 1999). An additional 200 cells were scored if mosaicism was detected. All the samples were analysed in duplicate on a blinded basis. In all mosaic cases, FISH results were reconfirmed in a second independent sample.

Polymerase chain reaction for sex determining region on Y gene

Genomic DNA was isolated from 5 ml peripheral blood using phenol–chloroform extraction. The oligonucleotide primers used in this study for the sex determining region on Y (SRY) gene were custom synthesized by Gibco BRL (USA). The primer sequences for the external and nested polymerase chain reaction (PCR) were: external primer forward, 5GAATATTCCCGCTCTCCGGA3; external primer reverse, 5GTACAACCTGTTGTCCA3; nested primer forward, 5CAG

TGTGAAACGGGAGAAAACAGT3; nested primer reverse, 5CTTCCGACGAGGTCGATACTTATA3.

PCR was performed on the genomic DNA according to the protocol described previously (Modi *et al*., 2005). Amplification using the external primers was carried out using the following cycling conditions: 94°C for 1 min, 53°C for 2 min, 72°C for 2 min for 30 cycles, and final extension at 72°C for 5 min. The first round PCR products were diluted 1:100 in distilled water and 2.5 μl of the diluted products was amplified using nested primers using the same PCR mix using the following cycling conditions: 94°C for 1 min, 65°C for 1 min and 72°C for 1 min for 25 cycles, and final extension at 72°C for 5 min. The products (15 µl volumes) were run on 2.5% agarose gels, stained with ethidium bromide, and visualized and photographed under UV light.

Several precautions were taken to avoid false-positive results in PCR. Frequent changing of gloves during DNA extraction and other PCR procedures and setting up the PCR reaction in a hood made the contamination with male cells less likely; the PCR mix was always prepared by a female operator. Pre- and post-PCR workplaces were always separated so that carry-over contamination of amplified DNA sequences to new PCR reaction could be avoided. Each set of PCR reaction included a positive control (male DNA), negative control (female DNA) and a template-free control (water in place of DNA) for early detection of contamination or PCR failure. Using the necessary precautions, amplification of SRY was not detected in any of the 10 normal female samples even after 40 cycles of nested PCR (not shown). All samples were analysed twice in separate experiments to confirm the results; the positive cases were checked for the third time when a female operator carried out all the operations independently.

Results

Cytogenetics

The cytogenetic findings of the cases analysed for the present study are summarized in **Table 2**. As evident, most of the cases with clinical suspicion of Turner syndrome had the 45,X karyotype, with or without mosaicism. Two cases had structural abnormalities of the X chromosome. Most cases with primary or secondary amenorrhoea had a normal karyotype; the most common cytogenetic aberration observed was mosaicism with 45,X cells.

FISH analysis

Using specific probes for two different regions of the Y chromosome (centromere and Yq), signals of appreciable intensities were detected in the interphase nuclei of cells obtained from male controls (**Figure 1**), no signals were observed in cells obtained from 10 females. Amongst the 73 patients analysed with both the probes in mononuclear cells, 11 patients had Y chromosome detected in 1–40% of cells. Of the variety of karyotypes possible with Y chromosome mosaicism as detected by FISH: X0/XY mosaicism was detected in three cases, XX/XY mosaicism in three cases, two cases had X0/XX/XY mosaicism, and three cases had X0/XX/XXX/XY mosaicism. In one case of primary amenorrhoea, the XXY genotype was observed only with the Yq probe (but not with the centromere). Combination of molecular and cytogenetic analysis revealed a 46,XX t(Yq:15) genotype (not shown). Representative photographs of some of these patterns are shown in **Figure 1**.

PCR for *SRY* gene

SRY gene PCR was performed in DNA obtained from peripheral blood cells only. In all the cases, prior to *SRY* gene PCR, the quality of genomic DNA was assessed by amplification of the autosomal housekeeping gene (glyceraldehyde-3-phosphate dehydrogenase) to avoid false-negative results. All samples showed an amplification band of the expected size (not shown). For SRY PCR, as expected, the external set of primers gave a band of 423 bp; the nested primers gave a band of 275 bp when male genomic DNA was used as template; no amplification products were detected when female genomic DNA was used as the template (**Figure 2**).

To determine the sensitivity of *SRY* gene PCR, serial dilution of male genomic DNA with female genomic DNA was used as a template. Using 0.3 µg genomic DNA, the amplification band was detected at a dilution ranging from 1:10 to 1:100 (male to female ratio), which is the equivalent of 10 male cells in 100 female cells (10% mosaicism). An additional 25 cycles of nested PCR detected male DNA at a dilution up to $1:10^{-9}$ that is equivalent to one male cell in 100,000 female cells (data not shown).

PCR was next performed on genomic DNA obtained from blood cells of all 73 patients. Using the external primers, a band of expected size was detected in three cases (**Figure 2A**).

Table 2. Cytogenetic findings in the patients included in the present study.

Karyotype	*Turner syndrome*	*Primary amenorrhoea*	*Secondary amenorrhoea*
45,X	6	0	0
45,X/46,XX	17	10	3
45,X/46,XX/47,XXX	0	1	0
45,X/47,XXX	0	0	0
46,X del(Xq)	1	0	0
46,X i(Xq)	1	0	0
46,XX	0	14	20
Total	25	25	23

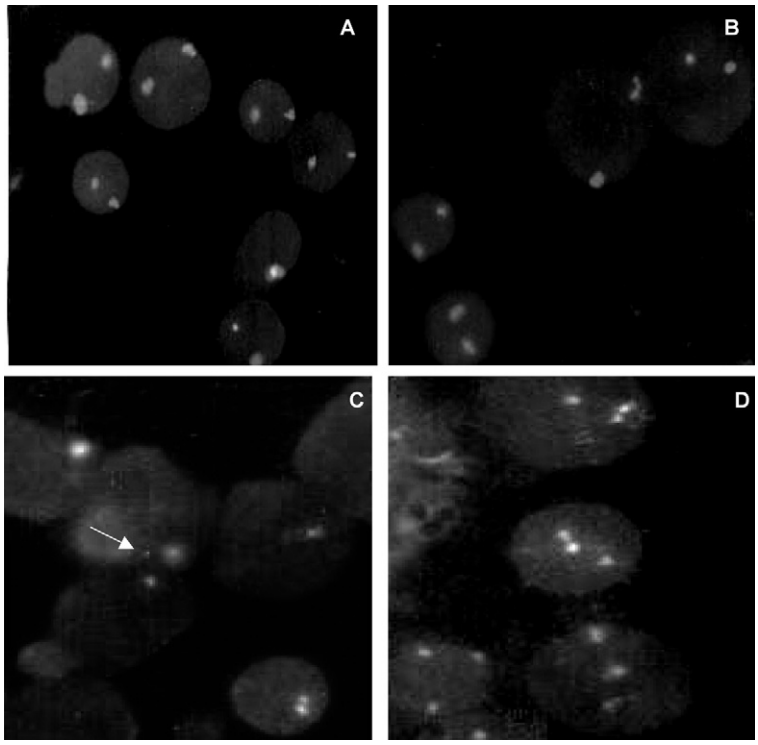

Figure 1. Interphase fluorescence in-situ hybridization (FISH) in mononuclear cells of patients with Turner syndrome and amenorrhoea. (**A**) FISH using XY centromeric probe in male cells. The green signal corresponds to the centromere of the X chromosome, red signals correspond to centromere of the Y chromosome. (**B**) Patient with primary amenorrhoea with XX/XY mosaicism. (**C**) XY cell (arrow) in a case of Turner syndrome showing X0/XY mosaicism. (**D**) XXY genotype in a patient with primary amenorrhoea. The probe detects the q arm of the Y chromosome (green signal); the centromere of X chromosome is labelled in red. Original magnification ×100. (Colour figure is available at http://www.rbmonline.com/Article/2741 but readers must be registered and subscribed to view.)

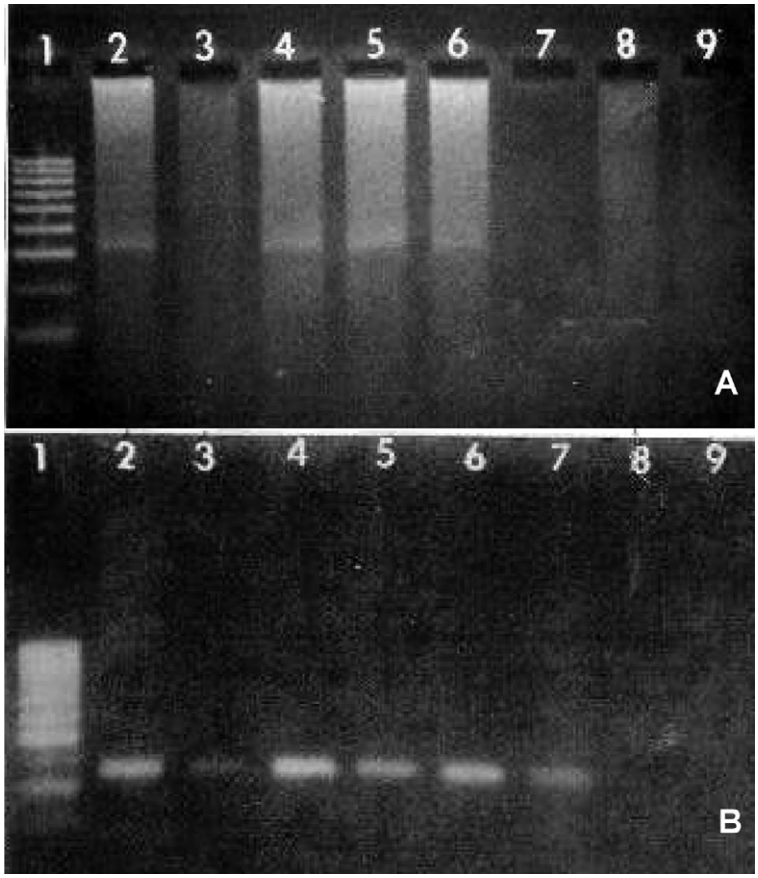

Figure 2. Sex determining region on Y (*SRY*) gene polymerase chain reaction (PCR) in cases of Turner syndrome and amenorrhoea. (**A**) PCR using external primers; (**B**) PCR using nested primers. Lanes: 1, 100-bp ladder; 2, male genomic DNA (positive control); 3–7, patient DNA; 8, female genomic DNA (negative control); 9, water control (PCR control).

However, using nested primers two additional patients were also detected as SRY positive (**Figure 2B**) indicating very low-grade mosaicism in these patients.

Combination of FISH and PCR results

Table 3 compares the FISH and *SRY* gene PCR results obtained in this study. A total of 13 cases had Y chromosome detected by FISH and PCR (including one detected in gonadal cells only). Of the 11 cases, intact Y chromosome was detected only in two cases; different regions were detected in the remaining patients. The centromere for the Y chromosome was detected in 10 cases; presence of the region encompassing only the q arm was observed in two cases; presence of only the *SRY* gene (p arm) was observed in one case. Repeated FISH in mononuclear cells of this patient failed to detect any cell with Y-chromosome centromere or q arm.

Screening for Y chromosome in buccal cells and gonadal cells

Buccal cells from 40 patients (including all 12 Y-chromosome-positive cases) were evaluated by FISH. The distribution of the patients according to the phenotype is given in **Table 4**. As expected, 11 of the 12 cases having Y-chromosome mosaicism in mononuclear cells (detected by FISH) had the same anomaly in buccal cells; no Y-chromosome signals were detected in the patient that was only SRY gene positive. The remaining 28 cases that were negative for Y-chromosome sequences in peripheral blood were also negative in buccal cells.

Gonadal cells obtained from 11 patients were screened by FISH. All except one had the same genotype as observed in peripheral blood and were negative for Y-chromosome sequences. Interestingly, in one case, approximately 2% of gonadal cells had the Y chromosome. The gonadal cell genotype was X0/XX/XY and the mosaicism was detected using both the centromeric and Yq probes. Repeated FISH and PCR in two independent blood samples from the same patient failed to detect any Y- chromosome mosaicism.

Gonadal histology

Y-chromosome mosaicism was detected in 13 of the 73 cases (18%): 16% of Turner syndrome cases, 28% of cases with primary amenorrhoea and 9% of cases with secondary amenorrhoea had Y-chromosome mosaicism (**Table 4**). Of these 13 cases, seven underwent prophylactic surgery to remove the gonads; biopsies from three additional patients (without any Y chromosome) were histologically examined by a trained pathologist. The results (**Table 5**) revealed presence of stromal tissue in eight cases. A few primordial follicles were observed in two cases. In one case with Y chromosome mosaicism (XX/XY genotype and Y chromosome in 40% of cells), the biopsy tissue showed histology resembling gonadoblastoma. Marker studies could not be undertaken in any cases for molecular phenotyping.

Discussion

Detection of Y-chromosome mosaicism in cases of gonadal dysgenesis is of clinical value owing to its association with gonadal malignancy. In the present study, Y chromosome mosaicism was detected in ~18% of cases with Turner syndrome and amenorrhoea, and 14% (1/7) of cases developed gonadoblastoma.

Several studies have evaluated the occurrence of Y chromosome mosaicism in Turner syndrome; the results of these studies are highly controversial. No Y chromosome mosaicism was observed in 12 45,X patients using Southern blotting with Y-specific probes that can detect one abnormal cell in 100 normal cells (Ostrer and Clayton, 1989). Using PCR with similar sensitivity, Medlej *et al.*

Table 3. Detection of Y chromosome loci by fluorescence in-situ hybridization and polymerase chain reaction.

Locus	*Patient no.*												
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*[a]
SRY	+	+	+	+	–	–	–	–	–	–	–	+	ND
Centromere	+	+	+	+	+	+	+	+	+	–	–	–	+
Yq	+	+	–	–	+	+	+	+	+	+	+	–	+
Clinical diagnosis	PA	PA	TS	TS	TS	TS	PA	PA	SA	SA	PA	PA	PA

[a]Detected only in gonadal cells.
ND = not done; PA = primary amenorrhoea; SA = secondary amenorrhoea; SRY = sex determining region on Y; TS = Turner syndrome.

Table 4. Comparison of Y chromosome mosaicism in blood, buccal and gonadal cells.

Clinical phenotype	*Y mosaics in blood*	*Y mosaics in buccal cells*	*Y mosaics in gonads*	*Total (%)*
Turner syndrome	4/25	4/15	1/2	4/25 (16)
Primary amenorrhoea	7/25	7/12	5/7	7/25 (28)
Secondary amenorrhoea	1/23	1/13	1/2	2/23 (9)

Table 5. Comparison of gonadal histology and fluorescence in-situ hybridization results in cases of Turner syndrome, primary amenorrhoea and secondary amenorrhoea.

Patient no.	*Clinical diagnosis*	*FISH results*	*Histology*
1	TS	X0/XX	Stroma only
2	TS	X0/XY	Stroma only
3	PA	X0/XX	Stroma only
4	PA	X0/XX	Stroma only
5	PA	X0/XY	Stroma only
6	PA	XX/XY	Stroma only
7	PA	XX/XY	Stroma only
8	PA	X0/XX/XY	Gonadoblastoma
9	PA	XXY t(Yq:15)	Few primordial follicles
10	SA	X0/XX/XXX	Few primordial follicles
11	SA	X0//XX/XXX/XY	Stroma only

FISH = fluorescence in-situ hybridization; PA = primary amenorrhoea; SA = secondary amenorrhoea; TS = Turner syndrome.

(1992) identified only one 45,X patient out of 40 as Y positive; none were detected in another study analysing 15 patients (Witt *et al.*, 1993). Kocova *et al.* (1993) improved the sensitivity of PCR further by Southern blotting the PCR products to detect one aberrant cell in 100,000 normal cells and reported a frequency as high as 40%. However, using nested PCR with a detection limit similar to that of Kocova *et al.* (1993), Binder and his group (1995) detected Y-positive Turner syndrome at a frequency of only 4% using the *SRY* gene as a marker; none were detected for the other two Y-chromosome-specific markers. In contrast, Pastalis *et al.* (1998) reported a frequency of 24% using PCR for 12 markers on the Y chromosome. In the present study, using a strategy similar to Binder *et al.* (1995) (nested PCR with the same sensitivity) coupled with FISH, the overall frequency of Y chromosome mosaicism in Turner syndrome was found to be 16%. These results contrast with those of Kocova *et al.* (1993) and Binder *et al.* (1995), but are similar to Gravholt *et al.* (2000) who reported Y chromosome mosaicism in nearly 12% of Turner syndrome cases. At present the reason for such controversial data is unclear; differences in the method used, regions analysed and selection criteria of the patients could be some of the possible causes; geographical or racial variations also can not be ruled out.

Currently, the frequency of Y chromosome mosaicism in cases other than Turner syndrome is not established. Conventional metaphase analysis has failed to identify Y chromosome mosaicism in any of the cases with primary or secondary amenorrhoea, oligomenorrhoea and premature ovarian failure (Turner *et al.*, 1970; Heine *et al.*, 1972; Temocin *et al.*, 1997). Interestingly, a recent study conducted on infertile couples revealed that sex chromosome but not autosomal abnormalities are more often detected in males compared with females (Kayed *et al.*, 2006), indicating that sex chromosome abnormalities may be a rare occurrence in infertile females. However, in this study, using a combination of FISH and PCR, nearly 28% of females with primary and 9% of cases with secondary amenorrhoea had occult Y-chromosome mosaicism. To the best of our knowledge these types of cases have not been evaluated previously for the presence of the Y chromosome using molecular methods; the present study underscores the need to evaluate these cases in more detail and assess the frequency of Y-chromosome mosaicism in female patients with amenorrhoea not presenting with frank Turner phenotype.

An interesting observation made in this study was the presence of different fragments of the Y chromosome in the patients. Only two of the 13 cases had all the three regions present (i.e. the centromere, *SRY* gene and the q arm), the rest of the cases had partial regions such as the q arm only or the SRY only. Similar observations have been made previously using PCR for different regions of the Y chromosome (Binder *et al.*, 1995; Pastalis *et al.*, 1998). This observation suggests that the Y chromosome when present is not always intact in most cases. Thus, in a clinical setting it may be necessary to employ multiple markers and more than one technique to evaluate hidden Y chromosome mosaics.

Failure to detect mosaicism can often be misleading. Variable genotypes in multiple tissues have often been observed in Turner syndrome cases (Ameil *et al.*, 1996; Modi *et al.*, 1999). Thus the absence of the Y chromosome or its fragments in blood need not necessarily mean its absence in the gonads. This has been reported in a 17-month-old Turner syndrome case with clit0romegaly, having Y chromosome mosaicism only in the gonads but not in blood (Bisat *et al.*, 1993). More recently screening of Turner syndrome cases in multiple tissues also revealed the occurrence of hidden Y chromosome in almost 35% of cases (Bianco *et al.*, 2006). These observations illustrate the limitations of molecular analysis employing blood only and suggest that it may be necessary to examine multiple tissues to identify occult mosaics (e.g. buccal cells, urinary cells, fibroblasts) before arriving at any conclusion. To investigate this possibility, in the present study buccal cells of 40 patients, along with peripheral blood, were evaluated to detect the presence of the Y chromosome. None of the cases that were negative for Y chromosome sequences in blood had mosaicism in the buccal cells. In 11 cases, gonadal cells were also studied to detect tissue-specific mosaicism. In all the cases but one, concordance was observed between blood and gonadal biopsy FISH results. However, in an exceptional case of secondary amenorrhoea (age 25 years) who underwent a diagnostic gonadal biopsy, FISH detected the presence of the centromere and the q arm in nearly 2% of the gonadal cells. Histologically, this case had streak gonads with no morphological evidence of any germ cells or tumour. This observation underscores the need of a systematic search for occult Y-chromosome mosaicism in patients with amenorrhoea.

The important clinical application of the detection of Y chromosome mosaicism is for prophylactic management of gonadoblastoma. Several studies have suggested a high incidence of gonadoblastoma in Y-chromosome-positive Turner syndrome cases (Canto *et al*., 2004; Brant *et al*., 2006). To evaluate the incidence of gonadoblastoma in the Y-chromosome-positive cases in the present study, tissue biopsies available from seven Y-chromosome-positive and three Y-chromosome-negative cases were histologically examined. The results revealed the occurrence of a germ cell tumour in one of the seven cases carrying Y-chromosome sequences, corresponding to a frequency of ~14%. Although the number of cases analysed herein is low and our patients are relatively young (age range of seven patients is 12–25 years), the frequency observed was significantly lower than that reported previously (Manuel *et al*., 1976; Verp and Simpson, 1987; Canto *et al*., 2004; Bianco *et al*., 2006; Brant *et al*., 2006). Our results are supported by the observations of Gravholt *et al*. (2000) who reported the occurrence of gonadoblastoma in only 12% of Y-chromosome-positive Turner syndrome cases. Together, these observations suggest that the risk of occurrence of gonadoblastoma in many studies may have been overestimated perhaps due to selection bias. There exists a need for large multicentric unbiased studies to determine the frequency of gonadoblastoma in Y-positive patients.

In conclusion, the results of the present study demonstrate that along with Turner syndrome cases the frequency of Y-chromosome mosaicism is relatively high and these patients may be predisposed to development of gonadal tumours. However, the frequency of occurrence of gonadoblastoma appeared to be lower than that reported previously, underscoring the need for large multicentre studies to establish a common consensus for the necessity of screening Turner syndrome and primary/secondary amenorrhoea cases by molecular methods for routine clinical applications.

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