ELECTRONIC LETTER

A comparative study between infertile males and patients with Turner syndrome to determine the influence of sex chromosome mosaicism and the breakpoints of structurally abnormal Y chromosomes on phenotypic sex

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The Y chromosome is important for male development as it contains the sex determining gene *SRY*[1] and many spermatogenesis genes.[2] Structural abnormalities of the Y chromosome include rings, deletions, inversions, and dicentrics.[3 4] These types of abnormalities are common in infertile males (1.5%), especially those with azoospermia.[5 6] However, such rearrangements are unstable and an additional 45,X cell line is frequently present.[3] The 45,X cell line has been shown to influence phenotypic sex so that these chromosome constitutions may also be found in patients with ambiguous genitalia and in female patients with gonadal dysgenesis and Turner syndrome.[4 7] In fact, from cytogenetic studies about 4-6.2% of female Turner patients show Y chromosome mosaicism[8–10] irrespective of the presence of *SRY*.[4 11 12]

Mosaicism varies widely between tissues and accurate interpretation depends on the number of cells examined and the number and types of tissues studied.[13 14] It has been reported that phenotypic sex is strongly influenced by the percentage and distribution of Y chromosome containing cells in the gonads.[15 16] However, studies on gonadal tissue are hindered by the fact that it is rarely available for analysis and alternative, more easily accessible tissue is usually studied.

It has also been suggested that the structure of the Y chromosome may indirectly affect phenotypic sex. The repetitive sequences at the euchromatin/heterochromatin boundary of the Y chromosome long arm are thought to have an important stabilising role and loss of this region loses this effect, resulting in mosaicism with a 45,X cell line.[17] In dicentrics, which are the most common abnormality of the Y chromosome,[3] it has been suggested that the position of the q arm breakpoint in dicentric Yp chromosomes can influence Y chromosome stability. The more proximal the long arm breakpoint, the greater the instability of the dicentric Yp chromosome which results in a higher percentage of 45,X cells and a sex phenotype which is more likely to be female.[18]

In this study, we have used a combination of G banded cytogenetic analysis and FISH to compare levels of sex chromosome mosaicism in different tissues from infertile males and female Turner patients, with a similar mosaic karyotype. FISH and PCR were also used to characterise deletion breakpoints of abnormal Y chromosomes to see whether they were related to the stability of the Y chromosome and therefore indirectly influenced phenotypic sex through levels of 45,X mosaicism.

Key points

- The Y chromosome is important for male development as it contains the sex determining gene *SRY* and many spermatogenesis genes. Structural abnormalities of the Y chromosome include rings, deletions, inversions, and dicentrics.
- These types of abnormalities are common in infertile males (1.5%), especially those with azoospermia. However, such rearrangements are unstable and an additional 45,X cell line is frequently present.
- The 45,X cell line has been shown to influence phenotypic sex so that these chromosome constitutions may also be found in patients with ambiguous genitalia and in female patients with gonadal dysgenesis and Turner syndrome. In fact, from cytogenetic studies, about 4-6.2% of female Turner patients show Y chromosome mosaicism, irrespective of the presence of *SRY*.
- In this study we have used a combination of G banded cytogenetic analysis and FISH to compare levels of sex chromosome mosaicism in different tissues from infertile males and female Turner patients, with a similar mosaic karyotype. We aimed to establish the influence of sex chromosome mosaicism on resulting phenotypic sex

MATERIALS AND METHODS

Subjects

All patients were referred to the Clinical Cytogenetics Unit, University College London Hospital (UCLH) either from the Departments of Endocrinology or Uro-nephrology or the Assisted Conception Unit, University College London Hospitals Trust. The three infertile males (M75, M99, and M103) had azoospermia and were identified as part of a study of 103 infertile males[19] and the three Turner syndrome patients (CS, NT, HE) were identified during a previous study of 54 Turner patients.[20] Peripheral lymphocytes were obtained from all patients, gonadal tissue from Turner patients CS and NT, and buccal cells from Turner patient HE and infertile male M103. The clinical and gonadal features of all patients are summarised in table 1 (patient HE was an adult Turner patient and details of her paediatric phenotype were not available).

Cytogenetic methods

Cytogenetic results from PHA stimulated blood lymphocytes from all patients and from the gonadal tissue of patient CS are reported elsewhere.[19 20] In this study, metaphases were additionally obtained from the gonadal tissue of patient NT and were stained by a GTL banding method, using standard techniques. One hundred metaphase spreads were analysed from each patient, which should detect 3% mosaicism with 95% confidence.[13]

Table 1 Clinical and gonadal features recorded for each patient

	Patients					
	M75	M103	M99	NT	CS	HE
Clinical features						
Short stature	+	–	–	+	+	+
Hyperconvex nails	–	–	–	+	–	NR
Low posterior hairline	–	–	–	+	+	NR
Broad chest/widely spaced nipples	–	–	–	+	+	NR
Cubitus valgus	–	–	–	+	–	NR
High arched palate	–	–	–	–	+	NR
Low prominent ears	–	–	–	+	–	NR
Pigmented naevus	+	–	–	–	–	NR
Duplex kidney	–	–	–	–	+	NR
Webbed/short neck	–	–	–	–	+	NR
Ear infections	–	–	–	+	–	NR
Coarctation of the aorta	+	–	–	–	–	NR
Oedema	–	–	–	+	–	NR
Gonadal features						
Bilateral streak gonads	NA	NA	NA	+	–	+
Ovo-testis/streak gonad	NA	NA	NA	–	+	–
Gonadoblastoma	–	–	–	–	+	–
History of undescended testis	+	–	–	NA	NA	NA
Mullerian remnant	+	–	–	NA	NA	NA
Azoospermia	+	+	+	NA	NA	NA

+=present, –=absent, NR=not recorded, NA=not applicable.

Table 2 Locus, deletion interval, size of product, and annealing temperature of each STS from PCR multiplexes V-VIII and single primer pair PCR

PCR	STS	Locus	Deletion interval	Size of product (bp)	Ta (°C)	Reference
MV	sY95	DYS280	5H	303	62	30
	sY100	DYS196	5I4	111		30
MVI	sY115	DYS207	5M3	115	62	30
	sY128	DYS219	5Q3	228		30
	sY134	DYS224	6A4	301		30
MVII	sY106	DYS202	5K2	231	58	30
	sY113	DYS205	5M1	290		30
MVIII	sY118	DYS210	5M3	218	62	30
	sY126	DYS217	5Q1	323		30
Single	sY122	DYS213	5N4	201	62	30

Ta=annealing temperature, STS=sequence tagged site, bp=base pairs.

FISH

Results of FISH with biotinylated cosmid 378E (prepared at the Lawrence Livermore National Library)[21] are reported elsewhere for infertile males[19] and Turner syndrome patients[20] and were used to identify *SRY* and the adjacent part of the pseudoautosomal region (PAR1).

In this study, additional FISH with a commercial X/Y subtelomeric probe set (Cytocell –CY29/c8.2/1) was carried out according to the manufacturer's instructions to characterise the Yp breakpoints of dicentric Yq chromosomes. FISH was also used to study sex chromosome mosaicism on interphase cells using a commercial α-satellite probe mix (Vysis) for chromosomes 18, X, and Y (CEP 18 Spectrum Aqua, CEP X Spectrum Green, and CEP Y Spectrum Orange). Peripheral lymphocytes were analysed from all patients, cultured gonadal tissue from Turner patients CS and NT, and buccal cells from Turner patient HE and infertile male M75. The 18 α-satellite probe was only viewed where necessary to establish ploidy. Slides of peripheral lymphocytes and gonadal tissue were prepared by standard methods and buccal cells according to a method described elsewhere.[22] The FISH method used and visualisation has been previously outlined.[23]

For metaphase analysis, a minimum of five metaphases containing the structurally abnormal Y chromosome were examined for each probe. For interphase FISH analysis of peripheral lymphocytes and gonadal tissue, 500 counts were made on good quality non-overlapping cells and over 50 cells were scored for buccal tissue. Peripheral lymphocytes were also scored from two normal male sperm donors, used as controls.

PCR

DNA was extracted from 5 ml of peripheral blood using a previously described method[24] or a commercial kit (QIAmp). Both groups of patients have been analysed by PCR as part of larger studies to screen for Y chromosome microdeletions in infertile males[19] and to detect the presence of Y sequences in Turner syndrome.[20] In this study, additional primers were used in four multiplex reactions and one single primer reaction to characterise Yq breakpoints and details of these primers are summarised in table 2. The PCR method was carried out as previously described.[19] All PCR assays were carried out at least twice, and control reactions were also performed with DNA extracted from a normal male, from a normal female, and water.

RESULTS

Cytogenetics

The cytogenetic results are summarised in table 3. The percentage of metaphases with a Y chromosome ranged from 36-95% in infertile male patients and from 3-26% in Turner patients. The percentage of cells with a Y chromosome was

Table 3 G banded cytogenetic results from the peripheral lymphocytes of all patients and the gonadal tissue of patients NT and CS

Patient	Tissue	Karyotype	Reference
M75	Lymphocytes	45,X[64]/46,X,idic(Y)(q11.2)[36]	19
M103	Lymphocytes	45,X[6]/46,X,idic(Y)(q11.2)[94]	19
M99	Lymphocytes	45,X[5]/46,X,idic(Y)(p11.3)[95]	19
NT	Lymphocytes	45,X[97]/46,X,idic(Y)(q11.2)[3]	20
	Gonadal	45,X[94]/46,X,idic(Y)(q11.2)[6]	–
CS	Lymphocytes	45,X,inv(5)(p14q11.2)[94]/47,X,idic(Y)(p11.3) + idic(Y)(p11.3), inv(5)(p14q11.2)[6]	20
	Gonadal	45,X,inv(5)(p14q11.2) [74]/47,X,idic(Y)(p11.3) + idic(Y)(p11.3), inv(5)(p14q11.2) [25]/46,X,idic(Y)(p11.3), inv(5)(p14q11.2)[1]	20
HE	Lymphocytes	45,X[92]/46,X,del(Y)(q11.2)[8]	20

Table 4 Interphase FISH results from all tissues from both sets of patients

	Signal number (%)							
Patients	Y	X	XY	XX	XXY	XYY	XYYYY	No of cells
Control male 1		1 (0.2)	499 (99.8)					500
Control male 2			496 (99.2)	2 (0.4)		2 (0.4)		500
M75		212 (42.4)		1 (0.2)		286 (57.2)	1 (0.2)	500
M99		39 (7.8)				459 (91.8)	2 (0.4)	500
M103		52 (10.4)				447 (89.4)	1 (0.2)	500
M103 b						54 (80.6)	12 (17.9)	67
NT		476 (95.2)		1 (0.2)		23 (4.6)		500
NT g		454 (90.8)		4 (0.8)	1 (0.2)	41 (8.2)		500
CS	1 (0.2)	433 (86.6)				4 (0.8)	62 (12.4)	500
CS g		296 (64.4)		2 (0.4)	1 (0.2)	13 (2.8)	148 (32)	500
HE		464 (90.8)	46 (9.2)					500
HE b		80 (80)	20 (20)					100

g=gonadal tissue, b=buccal mucosa, rest=lymphocytes, percentages in brackets.

found to be higher in the gonadal tissue than the peripheral lymphocytes for both Turner patients, but was still less than 30%. One metaphase out of 100 analysed from the gonadal tissue of Turner patient CS was found to have only one idic(Yq) chromosome compared to the 25 metaphases with two. FISH analysis confirmed the validity of this one cell.

PCR

Previous cytogenetic, FISH, and PCR analyses have shown that infertile males M75 and M103 and Turner patient NT have a cell line containing an idic(Yp) chromosome, and Turner patient HE has a cell line containing a del(Yq) chromosome.[19 20] In this study, PCR with additional primer pairs was used to refine the Y chromosome q arm breakpoints. These results are summarised as part of fig 1. Sequence tagged sites (STSs) have previously been assigned to Y chromosome deletion intervals.[25 26] Patients M75, M103, and NT were found to have breakpoints in Yq between Y chromosome STS sY118 (5M3) which was present and sY122 (5N4) which was absent in all three patients. Patient HE was found to have a more proximal Yq breakpoint between Y chromosome STSs sY100 (5I4) and sY106 (5K2).

FISH

Metaphase analysis

Our previous studies indicated that both Turner patient CS and infertile male M99 have a cell line containing an idic(Yq) chromosomes with two copies of *SRY*.[19 20] In this study the Yp subtelomeric probe (CY29) was found to be absent from the dicentric Yq chromosomes in both patients. The Yq subtelomeric probe (c8.2/1) was present at either end of the abnormal Y chromosomes and served as a control. These results are summarised as part of fig 1. The Yp breakpoint in both cases was therefore found to be between cosmid 378E specific for *SRY* and the adjacent part of PAR1 and the Yp subtelomeric probe (CY29). *SRY* and the Yp subtelomeric probe are 2.5 Mb and 100-300 kb from the Yp telomere, respectively.

Interphase analysis

Interphase FISH analysis of both groups of patients and controls are summarised in table 4. The percentage of interphase cells from peripheral lymphocytes with a Y signal ranged from 57.7-91.8% in infertile males and from 4.6-12.4% in Turner patients. Results from buccal and gonadal tissues gave higher levels of Y chromosome mosaicism than in the blood in all cases, but no higher than 35% for the Turner patients. Results from the control males indicated that any result found in less than approximately 1% of cells was not significant; therefore the majority of patients were found to have two cell lines, a 45,X cell line and a cell line with one X and an abnormal Y chromosome.

Infertile male patient M75, one exception, was found to have an additional cell line in the buccal cells with one X and four Y signals, thought to correspond to a 47,X,idic(Yp)+ idic(Yp) cell line. In the lymphocytes only 1/500 (0.2%) cells had been observed with this fluorescent pattern, which had been considered insignificant, but in view of the buccal cell results may represent this cell line at a very low level of mosaicism in the blood. In addition, a 45,X cell line was not recorded from the buccal mucosa of this patient but may have gone undetected owing to fewer cells being available for analysis. In the blood, Turner patient CS was found to have a second cell line with two idic(Yq) chromosomes. However, the FISH results from the gonadal tissue of this patient confirmed the presence of a third cell line with only one dicentric Yq, which was also seen in one G banded metaphase analysed from this tissue. Only 4/500 (0.8%) of interphase cells from the peripheral lymphocytes gave

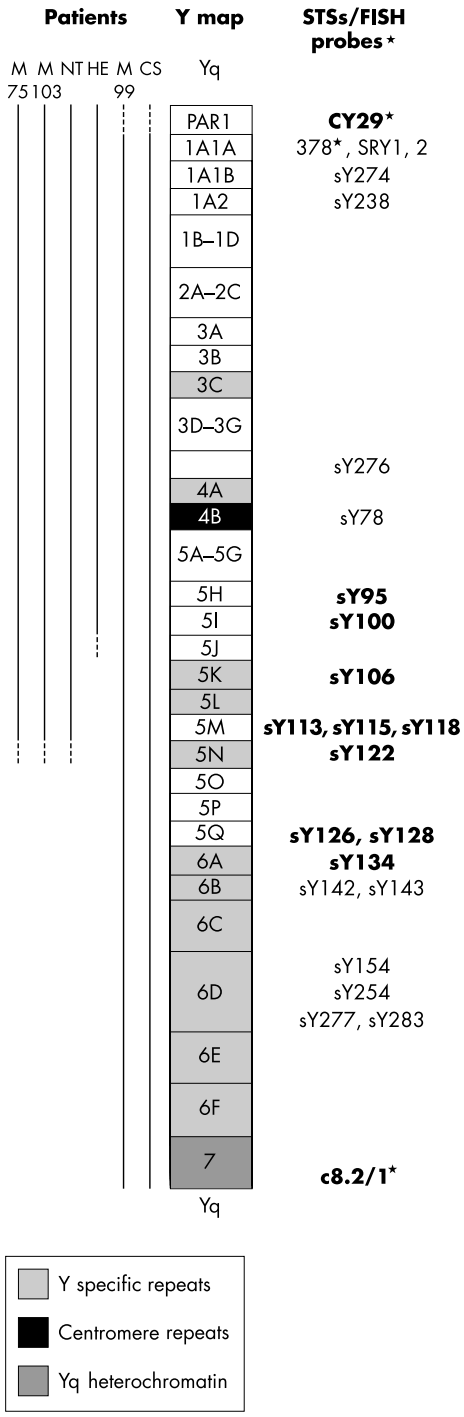

Figure 1 Y chromosome map based on that described previously.[25 31] Lines representing abnormal Y chromosomes are shown to the left of the Y chromosome map where a solid line represents Y sequences present, a broken line represents the region of the breakpoint, and a blank represents the extent of the deletion. Primers and FISH probes are shown to the right of the Y map with those used in this study in bold, others were used elsewhere.[19 20]

this result but again may represent the cell line at a very low level of mosaicism in the blood.

DISCUSSION

This study aimed to establish the influence of sex chromosome mosaicism on resulting phenotypic sex. Peripheral lymphocytes were examined for mosaicism in all patients because of their ease of accessibility and culture. Using standard cytogenetic methods, the levels of Y bearing cells were 36-95% in infertile males and 3-8% in Turner patients. These levels were similar when this tissue was examined by interphase FISH analysis, finding 57.7-91.8% and 4.6-12.4% respectively. Much higher percentages of Y bearing cells were therefore found in the male patients. Results from the blood of patient M75 gave the lowest percentage of Y bearing cells seen in a phenotypic male, with 36% after cytogenetic analysis and 57.7% after interphase FISH analysis. The latter probably gave a more accurate assessment of mosaicism levels owing to the greater number of cells examined. The higher percentage of 45,X bearing cells in M75 compared to the other males may explain the presence of Turner features in this patient but suggests that they were not at a high enough level to result in a female phenotype.

Blood is derived from the mesoderm but does not necessarily reflect the germ cells, which arise extragonadally in connection with the endoderm.[15] Comparison of different tissues in this study confirmed that there are variations in levels of mosaicism, with Y bearing cells being higher in other tissues than in the blood.

It was possible to examine gonadal tissue from two Turner patients (NT and CS) since gonadectomies had been performed as a preventative measure against the development of gonadoblastoma. The levels of Y bearing cells were seen to be at least twice as high in the gonads compared to the blood, with higher percentages again being found with interphase FISH. An additional cell line was also found after examination of the gonadal tissue of patient CS, presumably formed as a result of mitotic non-disjunction of the unstable dicentric Y chromosomes.

Gonadal tissue was not available from the other patients in this study. Where possible, buccal cells were examined as a second tissue because they were easily obtained and derive from a different cell lineage, the ectoderm. For Turner patient HE, the percentage of Y bearing cells was found to be twice as high after examination of the buccal mucosa cells, suggesting that there were significant differences in levels of mosaicism between tissues in this patient. The investigation of buccal mucosa also led to the discovery of an additional cell line in infertile male M103.

Overall, our mosaicism studies suggested that analysis of the blood gives a guide to levels of mosaicism in relation to phenotypic sex even though it is not necessarily identical to mosaicism in the gonads. In our patients, much higher percentages of Y bearing cells were found in the phenotypic males. It appeared that the percentage of 45,X cells in the female Turner patients resulted in the *SRY* gene being expressed below a critical threshold in the gonadal ridge of these females so that development along the male pathway did not occur. In order to predict the percentage of 45,X cells required for a patient to be phenotypically female rather than male, analysis of multiple tissues from many more cases will be necessary. In general, to obtain an accurate picture of mosaicism in terms of percentages and number of cell lines, analysis of more than one tissue from different cell lineages is required. Interphase FISH analysis appeared to be a simple method of analysing larger numbers of cells and could also be used to analyse cells from non-dividing tissues such as the buccal mucosa.

Our study also confirmed that dicentrics are a common structural rearrangement of the Y chromosome in mosaic patients. Minimal areas of euchromatin appeared to be deleted, which has also been reported previously.[27] We also examined the breakpoints of structurally abnormal Y chromosomes to see whether they had any effect on chromosomal stability, hence influencing levels of mosaicism and resulting phenotypic sex. Infertile male M99 and Turner patient CS both had dicentric Yq chromosomes and were found to have breakpoints between cosmid 378E and the Yp subtelomeric probe (CY29). However, the percentage of cells with the dicentric Yq

chromosome was markedly higher in male patient M99 and it appears that the position of the p arm breakpoint did not influence the stability of the dicentric Yq chromosome. The breakpoint is similar to that found in two Turner syndrome patients with dicentric Yq chromosomes described previously,[27] confirming that there are common Y chromosome p arm breakpoints susceptible to the formation of dicentric Yq chromosomes. These breakpoints appear to be within PAR1, which is where recombination occurs between the sex chromosomes, making it a hotspot for breakage and reunion.

It has been proposed that dicentric Yp chromosomes are less stable the more proximal the q arm breakpoint, resulting in higher levels of 45,X cells and a possible female phenotype.[17 18] In our study, the q arm breakpoint of the dicentric Yp chromosomes was between STS sY118 (5M3) and sY122 (5N4) in patients of both sexes, in distal interval 5. Again the cell lines containing the dicentric Y chromosome were at much higher levels in the phenotypic male patients, which suggested that the position of the q arm breakpoint in dicentric Yp chromosomes also did not appear to influence Y chromosome stability. The breakpoint in our patients is similar to a previously described case of a Turner patient with a dicentric Yp chromosome in which the breakpoint was determined to be between Y chromosome intervals 5J and 5Q.[27] Other studies of Turner patients with a cell line containing a dicentric Yp chromosome have reported a q arm breakpoint in what appears to be a common region of distal Y chromosome deletion interval 6, close to the heterochromatic block.[12 27] Both this position and the breakpoints found in our patients occur in the Y specific repeat regions of Yq.[25] However, the breakpoint seen in Turner patient HE, who had a terminal deletion of the Y chromosome q arm was in a more proximal region of Yq outside the Y specific repeats. We therefore propose that multiple areas of Y specific repeat sequences along the Y chromosome q arm are susceptible to breakage and reunion and in these cases formation of dicentrics. This is substantiated by reports that common Yq microdeletions found in infertile males are bounded by Y repeat regions, thought to serve as substrates for homologous recombination.[28 29]

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