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

C R Quilter, N Nathwani, G S Conway, R Stanhope, D Ralph, G Bahadur, P Serhal, K Taylor, J D A Delhanty

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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.

Mosaicism varies widely between tissues and accurate interpretation depends on the number of cells examined and the number and types of tissues studied. It has been reported that phenotypic sex is strongly influenced by the percentage and distribution of Y chromosome containing cells in the gonads. 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. In dicentrics, which are the most common abnormality of the Y chromosome, 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.

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.

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 and the three Turner syndrome patients (CS, NT, HE) were identified during a previous study of 54 Turner patients. 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. 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.
FISH

Results of FISH with biotinylated cosmid 378E (prepared at the Lawrence Livermore National Library) were reported elsewhere for infertile males and Turner syndrome patients and were used to identify SRY and the adjacent part of the pseudo-autosomal 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.

The FISH method used and visualisation has been previously outlined. 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 or a commercial kit (QIAGen). Both groups of patients have been analysed by PCR as part of larger studies to screen for Y chromosome microdeletions in infertile males and to detect the presence of Y sequences in Turner syndrome.

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. 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

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### Table 1

Clinical and gonadal features recorded for each patient

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

+=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

<table>
<thead>
<tr>
<th>PCR</th>
<th>STS</th>
<th>Locus</th>
<th>Deletion interval</th>
<th>Size of product (bp)</th>
<th>Ta (°C)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>MV</td>
<td>sY95</td>
<td>DYS280</td>
<td>5H</td>
<td>303</td>
<td>62</td>
<td>30</td>
</tr>
<tr>
<td>MV</td>
<td>sY100</td>
<td>DYS196</td>
<td>5A</td>
<td>111</td>
<td>62</td>
<td>30</td>
</tr>
<tr>
<td>MVI</td>
<td>sY115</td>
<td>DYS207</td>
<td>5M3</td>
<td>115</td>
<td>62</td>
<td>30</td>
</tr>
<tr>
<td>MVI</td>
<td>sY128</td>
<td>DYS219</td>
<td>5G3</td>
<td>228</td>
<td>62</td>
<td>30</td>
</tr>
<tr>
<td>MVI</td>
<td>sY134</td>
<td>DYS224</td>
<td>5A4</td>
<td>301</td>
<td>62</td>
<td>30</td>
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<tr>
<td>MVII</td>
<td>sY106</td>
<td>DYS202</td>
<td>5K2</td>
<td>231</td>
<td>58</td>
<td>30</td>
</tr>
<tr>
<td>MVII</td>
<td>sY113</td>
<td>DYS205</td>
<td>5M1</td>
<td>290</td>
<td>58</td>
<td>30</td>
</tr>
<tr>
<td>MVII</td>
<td>sY118</td>
<td>DYS210</td>
<td>5M3</td>
<td>218</td>
<td>62</td>
<td>30</td>
</tr>
<tr>
<td>MVII</td>
<td>sY126</td>
<td>DYS217</td>
<td>5G1</td>
<td>323</td>
<td>62</td>
<td>30</td>
</tr>
<tr>
<td>Single</td>
<td>sY122</td>
<td>DYS213</td>
<td>5N4</td>
<td>201</td>
<td>62</td>
<td>30</td>
</tr>
</tbody>
</table>

Ta=annealing temperature, STS=sequence tagged site, bp=base pairs.
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. 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. Patients M75, M103, and NT were found to have breakpoints in Yq between Y chromosome STSs sY118 (5M3) which was present and sY122 (5N4) which was absent in all three patients. Patient HE was found to have breakpoints in Yq between Y chromosome STSs 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 sY118 (5M3) 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 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.

**Table 4**

> | Patients         | YXYXXYYXYYYY No of cells |
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Control male 1</td>
<td>1 (0.2)</td>
</tr>
<tr>
<td>Control male 2</td>
<td>212 (42.4)</td>
</tr>
<tr>
<td>M75</td>
<td>45, X[64]/46, X,idic(Y)(q11.2)[36]</td>
</tr>
<tr>
<td>M103</td>
<td>45, X[65]/46, X,idic(Y)(q11.2)[94]</td>
</tr>
<tr>
<td>M99</td>
<td>45, X[65]/46, X,idic(Y)(p11.3)[95]</td>
</tr>
<tr>
<td>NT</td>
<td>45, X[97]/46, X,idic(Y)(q11.2)[3]</td>
</tr>
<tr>
<td>Gonadal</td>
<td>45, X[94]/46, X,idic(Y)(q11.2)[6]</td>
</tr>
<tr>
<td>CS</td>
<td>45, X,inv(5)q14(1.2[94]/47, X,idic(Y)(p11.3) + idic(Y)(p11.3), inv(5)p14q11.2)[6]</td>
</tr>
<tr>
<td>HE</td>
<td>44, X,del(Y)(q11.2)[8]</td>
</tr>
</tbody>
</table>

*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.
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. 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 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. 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, 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 than the more proximal q arm breakpoint, resulting in higher levels of 45,X cells and a possible female phenotype.\textsuperscript{15, 16} In our study, the q arm breakpoint of the dicentric Yp chromosomes was between STS sY118 (5M3) and sY12 (5M4) 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.\textsuperscript{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.\textsuperscript{17, 18} Both this position and the breakpoints found in our patients occurred in the Y specific repeat regions of Yq.\textsuperscript{15} 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 dicentric. 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.\textsuperscript{25, 26}

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Authors' affiliations

C R Quilter, K Taylor, The Galton Laboratory, University College London, Wolfson House, 4 Stephenson Way, London NW1 2HE, UK
N Nathwani, G S Conway, R Stanhope, Department of Endocrinology, University College London Hospitals Trust, London WC1, UK
D Ralph, Department of Uro-nephrology, University College London Hospitals Trust, London WC1, UK
G Bahadur, J D A Delhanty, Department of Obstetrics and Gynaecology, University College London Hospitals Trust, London WC1, UK
P Serhal, Assisted Conception Unit, University College London Hospitals Trust, London WC1, UK

Correspondence to Dr C R Quilter, Department of Pathology, University of Cambridge, Tennis Court Road, Cambridge CB2 1GP, UK, crq20@cam.ac.uk

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