X inactivation patterns in female monozygotic twins and their families

E Watkiss, T Webb, G Rysiecki, N Girdler, E Hewett, S Bundey

Abstract
X inactivation studies have been carried out on 22 pairs of female monozygotic twins, one set of female monozygotic triplets, and their mothers and singleton sisters, using the probe M27β. Forty-eight per cent of the twins, 55% of their mothers, and 42% of their singleton sisters showed skewed X inactivation. Two of the triplets and their mother had random X inactivation, while the third triplet showed skewed X inactivation. Their singleton sister was homozygous with M27β. Of the twins, six pairs showed skewed X inactivation in favour of the same X chromosome, one pair showed skewed X inactivation favouring opposite X chromosomes, in seven pairs one twin showed skewed X inactivation while her co-twin showed random X inactivation, and in eight pairs both twins were random. A higher frequency of skewed pattern of X inactivation was not observed in the monozygotic twins when compared to a series of non-twin females (mothers and singleton sisters) and, so, the results in this study do not lend support to the theory that skewed X inactivation predisposes to the twinning process.

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Manifestation of X linked disease in female heterozygotes is often thought to be the result of non-random X inactivation with or without a chromosomal aberration. Manifestation of disease in one member of a pair of female monozygotic twins has also been found in a variety of X linked disorders. These include Christmas disease, fragile X syndrome, colour vision deficiency, Hunter’s disease, Fabry’s disease, Aicardi syndrome, and muscular dystrophy. In all of these cases, the twins were discordant for manifestation of the X linked disorder and often the affected twin was more severely handicapped than was usual for a manifesting carrier. In seven cases there was non-random X inactivation in the affected twin. The difference in X inactivation of the X chromosomes such that the mutant homologue remained preferentially active provided an explanation for the discordance of disease manifestation found in the twins.

The discordance in manifestation of X linked disease that is sometimes seen in female monozygotic twins led Burn et al. to suggest that there may be a relationship between anomalies of X inactivation and the twinning process. They proposed that if X inactivation preceded twinning, the two types of cell population produced could become separated, so that an inactivated maternally derived X chromosome predominated on one side of the zygote and an inactivated paternally derived X on the other side. Separation of the two cell populations by twinning could then lead to one twin being asymptomatic and the other being affected. Alternatively the two cell populations could possibly re-epel each other and actually predispose to monozygotic twinning.

Nance suggested that asymmetrical splitting of the inner cell mass could lead to discordant expression owing to the sampling process associated with the mechanism. Any pre-existing inequalities within the embryo would be exaggerated by the division, leading to the development of discordant twins, whereby the affected twin has skewed X inactivation with the mutant gene being preferentially active and her co-twin having either random X inactivation or skewed X inactivation in favour of the non-mutant gene remaining preferentially active.

Obviously X inactivation cannot explain the occurrence of male monozygotic twins and so presumably the same mechanism that causes male twins could also produce female twins. X inactivation could represent an additional mechanism restricted only to females.

If X inactivation is an additional factor in female monozygotic twinning, then an excess of female monozygotic twins over male monozygotic twins would be expected. Burn et al. noted that such an excess was not obvious but did find that 70% of conjoined twins, resulting from very late twinning, were female. Similarly, James showed an increased percentage of female as opposed to male monozygotic twins with later times of placentation.

Generally twins are not half the size of singletons at the time of birth, in spite of the fact that they arise from an inner cell mass that contains approximately half the number of cells. Richards et al. suggested that catch up growth must occur. If X inactivation precedes twinning, then the catch up divisions may lead to an increase in the three dimensional mosaic patch size in adult twins, as was observed in the affected twin reported by Richards et al.

If the discordant expression of an X linked disease in some female monozygotic twins is related to opposite X inactivation, then this should be detectable in a percentage of normal female monozygotic twins not known to be carriers of an X linked disease.

This study used methylation patterns in a series of monozygotic female twins in order to determine whether there was any discordancy in their X inactivation profiles which may sup-
port the possibility of a relationship between X inactivation and the twinning process.

The probe M27β detects locus DXS255 at Xp11.2. There is, within this locus, a VNTR region with a heterozygosity of about 90% and also a CpG island containing restriction sites for the enzyme MspI. When cytosine residues within these restriction sites are methylated, they become resistant to cleavage by the methylation sensitive isochizomer HpaII. Thus active and inactive X chromosomes may be differentiated, since the cytosine residues of these sites are fully methylated, and so remain uncut by HpaII only on the active X chromosome.\(^9\)

**Methods**

Twenty-five adult apparently monozygotic twin pairs with mothers and singleton sisters who were all willing to participate in the study were ascertained through the Birmingham University twin register. All 100 females in these 25 families were visited at home; a pedigree was constructed and questions were asked about the birth of the twins and about subsequent health problems in them or their relatives. Ishihara colour tests were performed on the participants if there was a history of colour blindness in the family. In addition to these, one set of triplets, their mother, and a singleton sister were also investigated.

A total of 20 ml of blood was taken in potassium EDTA tubes and DNA was extracted from the lymphocytes using a 340A nucleic acid extractor (Applied Biosystems). DNA from the twins and the triplets was sent to Cellmark Diagnostics for zygosity studies. Only one of the pairs of twins was found to be dizygotic, using two multilocus and five single locus probes.

DNA (15 µg) from each subject was digested with PstI or AvaII. Digests were then reprecipitated, split into three parallel samples, and either (1) no further digest, (2) MspI digests, or (3) HpaII digests were performed. Products were electrophoresed on a 0.7% agarose gel and Southern blotted under standard conditions. Filters were hybridised with radiolabelled probe M27β. The autoradiographs obtained were studied by eye by two independent observers and also visualised using an LKB Ultrascan XL Laser densitometer. Relative intensities of the active X chromosome alleles were compared to determine the degree of X inactivation.

The active alleles were seen as an extra two bands in the HpaII + PstI digests which were not present in the MspI or PstI digests, but were located in the same position as the original bands present in the PstI digests. When PstI was used as the initial enzyme, the active and inactive X chromosome bands were 0.5 kb apart. Problems arose when the VNTR region of the two X chromosomes also differed by about 0.5 kb. A single active band then became superimposed upon the inactive band of the other X chromosome. This was overcome by repeating the digests with AvaII instead of PstI, since then the bands differ in size by only 0.3 kb.

Difficulties were encountered both in the definition and in the interpretation of what is meant by skewing and in making meaningful comparisons between the extent of non-randomness detected in this study and those reported previously. When studied by eye subjects were classed as having random, moderately skewed, or extremely skewed X inactivation. In combination with densitometry moderate skewing was categorised as a ratio of the two active band intensities greater than 65:35 and less than 80:20, and extreme skewing as ratios of 80:20 or above; these definitions correspond to those of Harris et al.\(^9\)

**Results**

One pair of twins proved to be dizygotic and were excluded from the study, one pair were homozygous with M27β, and one pair failed to give results owing to degraded DNA. No colour blind subjects were found among the females in this study. Results for the remaining twin families are shown in table 1. Twenty-one of 44 (48%) of the twins, 12/22 (55%) of their mothers, and 9/22 (42%) of their non-twin sisters showed either moderately skewed or extremely skewed X inactivation with the probe M27β, and 8/44 twins, 9/22 mothers, and 4/22 singleton sisters showed extremely skewed X inactivation. No preferential inheritance of one particular maternal X chromosome by twin pairs as opposed to singletons was noted, as out of 23 families the same X chromosome was inherited by the twins or triplets and their singleton sister 11 times and the opposite X chromosome 12 times.

A summary of the X inactivation profiles of each twin pair is shown in table 2. In two of the 22 twin pairs, skewing or extreme skewing was found in favour of the same X chromosome in both twins. In two pairs, one twin showed skewed X inactivation while the co-twin did not, while in the remaining pairs, there was no skewing in either twin at a level greater than 65:35. In the five twin pairs, where moderate skewing (65:35 only) was present, four pairs had the same X chromosome preferentially active and in only one pair was the maternal X preferred in one twin and the paternal X in the other.

**Table 1** X inactivation profiles of the twin pairs, their mothers, and their singleton sisters

<table>
<thead>
<tr>
<th></th>
<th>No moderately skewed</th>
<th>No extremely skewed</th>
<th>No random</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Twins</td>
<td>13 (30%)</td>
<td>8 (18%)</td>
<td>23 (52%)</td>
<td>44</td>
</tr>
<tr>
<td>Mothers</td>
<td>3 (14%)</td>
<td>9 (41%)</td>
<td>10 (45%)</td>
<td>22</td>
</tr>
<tr>
<td>Sisters</td>
<td>5 (23%)</td>
<td>4 (19%)</td>
<td>13 (36%)</td>
<td>22</td>
</tr>
</tbody>
</table>

**Table 2** X inactivation profiles of each twin member

<table>
<thead>
<tr>
<th>Type of X inactivation found in each twin pair</th>
<th>Nos</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skewed-skewed: same direction</td>
<td>6</td>
</tr>
<tr>
<td>Random-random</td>
<td>8</td>
</tr>
<tr>
<td>Concordant profiles</td>
<td>14</td>
</tr>
<tr>
<td>Skewed-skewed: opposite directions</td>
<td>1</td>
</tr>
<tr>
<td>Skewed-random</td>
<td>7</td>
</tr>
<tr>
<td>Discordant profiles</td>
<td>8</td>
</tr>
</tbody>
</table>
The triplets proved to be all monozygotic. Two of the triplet sisters and their mother showed random X inactivation, while the third showed moderately skewed X inactivation, and their non-triplet sister was homozygous with M27β. The maternally inherited allele was preferentially active in the triplet, who showed moderate skewing.

When the families were examined individually, there was no evidence to suggest that there may be a relationship showing preferential skewing towards the same chromosome remaining active in the mothers, the singleton sisters, or the twin pairs. Of the two twin pairs with concordant extreme skewing, in both pairs the paternally inherited homologue was preferentially active, whereas in the four singleton sisters who showed skewing, the maternal X chromosome was preferentially active three times and the maternal X once. We also looked to see if one particular X chromosome had a tendency to remain active when passed from one generation to the next. On 12 occasions the maternal X chromosomes were found to be non-randomly inactivated (65:35 or greater). The twin pair had inherited this preferentially active maternal homologue 6/12 times, inheriting the non-active homologue an equal number of times. The preferentially active homologue showed no tendency to remain active as it suffered random inactivation on seven occasions and became the preferentially inactive homologue four times when inherited by the twin pair. The singleton sister inherited the preferentially active maternal homologue 4/12 times and it remained so once, becoming randomly inactivated on two occasions and preferentially inactive once.

**Discussion**

The results indicate that there is not a significantly higher frequency of skewed patterns of X inactivation in monozygotic twins compared to non-twin females. The non-twin females in this study were the mothers and singleton sisters of the twin pairs. The data reported for a series of 42 normal females were also used for comparison. In addition, the data in this study do not show any familial tendency towards skewed patterns of X inactivation.

The results in this study do not lend support to the hypothesis that skewed X inactivation predisposes to the twinning process, as only 1/22 twin pairs showed any skewing in opposite directions while 6/22 showed concordant skewing.

Similar findings were reported by Goodship et al. and by Richards et al. Richards et al. studied the X inactivation profiles of 20 normal female monozygotic twin pairs; 18/20 (90%) of them showed concordancy for X inactivation patterns, while only 2/20 (10%) showed opposite skewed X inactivation patterns. They suggested that the concordancy could be the result of prenatal vascular anastomoses which may obscure any marked discordance in the X inactivation profiles of myeloid cells.

Concordant skewing has not been reported as often in female carrier twins as discordant skewing. Costa et al. studied a pair of twins who were discordant for Aicardi's syndrome, both of whom had random X inactivation in their lymphocytes and Goodship et al. found concordant skewing in a pair of monozygotic female twins who were discordant for Duchenne muscular dystrophy. Jorgenson et al. could not find any skewing of X inactivation in lymphocytes from twins discordant for colour vision deficiency, but did find skewing in the fibroblasts of the colour vision defective twin. They suggested that failure to find this skewed pattern in lymphocytes could be explained by the sharing of fetal circulation and exchange of haematopoietic precursor cells owing to prenatal vascular anastomoses.

The idea that vascular anastomoses can obscure any initial skewing in the lymphocytes is attractive. If X inactivation is more likely to lead to twinning with later developmental stages of the embryo, then presumably X inactivation is more likely to lead to the formation of monochorionic twins. If 80% of monozygotic twins are monochorionic and 85% of monochorionic twins have vascular anastomoses, then 68% of twins would be expected to be both monochorionic and have had prenatal vascular anastomoses present during development. This could explain at least some of the concordancy found in the lymphocytes of 64% of the twin pairs in this and other studies. Brown and Brown studied skin fibroblasts rather than lymphocytes in a series of 48 patients with pyruvate dehydrogenase deficiency and 20 female controls and found that 24% of their sample had X inactivation ratios greater than 80:20. Recent studies by Tan et al. have suggested that X inactivation is a dynamic process which occurs at different times in different tissues.

### Table 3. X inactivation studies in female MZ twins discordant for X linked disease

<table>
<thead>
<tr>
<th>Study</th>
<th>Disease</th>
<th>Method</th>
<th>Tissue</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tuckerman et al.</td>
<td>DMD</td>
<td>FISH</td>
<td>Lymphocytes and fibroblasts</td>
<td>Opposite skewing found in twins</td>
</tr>
<tr>
<td>Costa et al.</td>
<td>DMD</td>
<td>M27β</td>
<td>Lymphocytes</td>
<td>Random X inactivation found in both twins</td>
</tr>
<tr>
<td>Richards et al.</td>
<td>DMD</td>
<td>FISH</td>
<td>Lymphocytes</td>
<td>Opposite skewing found in twins</td>
</tr>
<tr>
<td>Zeeemer et al.</td>
<td>DMD</td>
<td>M27β</td>
<td>Lymphocytes</td>
<td>85% opposite skewing found in same twins</td>
</tr>
<tr>
<td>Lupski et al.</td>
<td>DMD</td>
<td>M27β</td>
<td>Lymphocytes and fibroblasts</td>
<td>Opposite skewing found in same twins</td>
</tr>
<tr>
<td>Jorgensen et al.</td>
<td>DMD</td>
<td>M27β</td>
<td>Lymphocytes</td>
<td>Opposite skewing found in same twins</td>
</tr>
<tr>
<td>Winchester et al.</td>
<td>DMD</td>
<td>M27β</td>
<td>Lymphocytes</td>
<td>Skewing found in affected skin, normal twin random</td>
</tr>
</tbody>
</table>

- Opposite skewing found in twins
- Random X inactivation found in both twins
- Opposite skewing found in twins
- 85% opposite skewing found in same twins
- Opposite skewing found in same twins
- Skewing found in affected skin, normal twin random
- Normal random
Therefore although we have not found any evidence of a relationship between X inactivation in lymphocytes and the twinning process this could be because initially skewed X inactivation has been masked by later vascular anastomoses. For this reason, Goodship et al. used umbilical cord DNA rather than lymphocyte DNA in their study.

We have reviewed the findings of our study along with those of others on monozygotic female twins together with observations on manifesting carriers of X linked disease (tables 3 and 4). In addition to the studies described, further singleton females have been found to manifest Xp21 muscular dystrophy along with skewing of their X chromosomes (Watkiss et al., unpublished data). Except for Xp21 muscular dystrophy, no strong association between twinning and skewing in lymphocytes emerges. It is noteworthy that one of the studies which detected a strong association between X linked disease and skewing of X chromosome inactivation used fibroblasts.

We would like to thank Action Research and the Endowment Fund of the United Birmingham Hospitals for financial support, the Department of Genetics at Birmingham University for information on their twin registries, and DNA laboratory at Birmingham Maternity Hospital for extraction of samples and valuable advice during the course of this study.

Table 4 Proportion of females displaying skewed X inactivation

<table>
<thead>
<tr>
<th>Degree of skewing</th>
<th>Healthy MZ twins (This study, Richards et al.1)</th>
<th>Goodship et al.2</th>
<th>Healthy siblings</th>
<th>Female carriers of X linked disease</th>
<th>ALD (n = 35)</th>
<th>MZ twins (Richards et al.1)</th>
<th>Controls (Goodship et al.2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.65 &lt; 0.80</td>
<td>13/44</td>
<td>8/30</td>
<td>14/26</td>
<td>0/12</td>
<td>0.07</td>
<td>0.12</td>
<td>0.08</td>
</tr>
<tr>
<td>Or moderate skewing</td>
<td>0/30</td>
<td>0/30</td>
<td>0/35</td>
<td>0/30</td>
<td>0.19</td>
<td>0.43</td>
<td>0.45</td>
</tr>
<tr>
<td>&gt; 0.80 or extreme skewing</td>
<td>8/44</td>
<td>11/86</td>
<td>13/42</td>
<td>0.01</td>
<td>0.57</td>
<td>0.03</td>
<td>0.07</td>
</tr>
<tr>
<td>Total skewed</td>
<td>21/44</td>
<td>25/40</td>
<td>21/44</td>
<td>6/12</td>
<td>0.76</td>
<td>0.46</td>
<td>0.50</td>
</tr>
</tbody>
</table>

*Only those with discordant patterns, therefore not fair comparison. †This study used methylation PCR +"eye balled" (no densitometry). ‡Using PGK, HPRT, or M273, using skin fibroblasts. ALD = adenoleucodystrophy. IP = incontinentia pigmenti. PDHEI def = pyruvate dehydrogenase deficiency.


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