X inactivation patterns in female monozygotic twins and their families

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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 M27 β .

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 M27 β . 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,¹ fragile X syndrome,^{2,3} colour vision deficiency,^{4,5} Hunter's disease,⁶ Fabry's disease,⁷ Aicardi syndrome,⁸ and muscular dystrophy.⁹⁻¹⁵

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^{2,3,5,6,10,13-15} 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*¹⁰ 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 repel each other and actually predispose to monozygotic twinning.

Nance¹⁶ 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*¹⁰ noted that such an excess was not obvious but did find that 70% of conjoined twins, resulting from very late twinning, were female. Similarly, James¹⁷ 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*¹³ 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,¹⁶ as was observed in the affected twin reported by Richards *et al*.¹³

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-

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port the possibility of a relationship between X inactivation and the twinning process.

The probe M27β detects locus DXS255 at Xp11.2.¹⁸ 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.¹⁹

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 Ultra-scan 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. One 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.*²⁰

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

	No moderately skewed	No extremely skewed	No random	Total
Twins	13 (30%)	8 (18%)	23 (52%)	44
Mothers	3 (14%)	9 (41%)	10 (45%)	22
Sisters	5 (23%)	4 (19%)	13 (58%)	22

Table 2 X inactivation profiles of each twin member

Type of X inactivation found in each twin pair	Nos
Skewed-skewed: same direction	6
Random-random	8
Concordant profiles	14
Skewed-skewed: opposite directions	1
Skewed-random	7
Discordant profiles	8

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 M27 β . 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 paternal 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 females²⁰ 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.*²¹ and by Richards *et al.*²² Richards *et al.*²² 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.*⁶ 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.*²¹ found concordant skewing in a pair of monozygotic female twins who were discordant for Duchenne muscular dystrophy. Jorgenson *et al.*⁵ 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 monochorionic²² and 85% of monochorionic twins have vascular anastomoses,²³ 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 in other studies. Brown and Brown²⁴ 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.*²⁵ 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

Study	Disease	Method	Tissue	Results
Tuckerman <i>et al.</i> ²	Fra(X)	BrdU	Lymphocytes	Opposite skewing found in twins
Burn <i>et al.</i> ¹⁰	DMD	Somatic cell hybridisation	Skin fibroblasts	Opposite skewing found in twins
Costa <i>et al.</i> ⁶	Aicardi's syndrome	M27 β	EBV transformed B lymphocytes	Random X inactivation found in both twins
Richards <i>et al.</i> ¹³	DMD	M27 β	Lymphocytes and fibroblasts	Opposite skewing found in twins
Zneimer <i>et al.</i> ¹⁵	DMD	FISH		85% opposite skewing found in same twins that Richards <i>et al.</i> ¹³ studied
Lupski <i>et al.</i> ¹⁴	DMD	M27 β	Lymphocytes	Skewing found in affected skin, normal twin random
Jorgensen <i>et al.</i> ⁵	Colour vision def	M27 β	Skin and lymphocytes	Opposite skewing found in the twin's skin, random in lymphocytes of both twins
Winchester <i>et al.</i> ⁶	Hunter's disease	M27 β	Lymphocytes and fibroblasts	Skewing found in affected twin in both tissues. Normal twin random

Table 4 Proportion of females displaying skewed X inactivation

Degree of skewing	Healthy MZ twins			Healthy singletons		Female carriers of X linked disease					
	This study	Richards et al ¹³	Goodship et al ²¹	This study (mothers + sisters)	Harris et al ²⁰ (controls)	ALD Watkiss et al ²⁶	IP Harris et al ²⁰	PDHEI def Brown and Brown ²⁴	Alport's Vetrie et al ²⁷		Muscular dystrophy Bushby et al ²⁸
									AS females	Unaffected relatives	
0.65 < 0.80 or moderate skewing	13/44 0.30	—	26/86 0.23	8/44 0.18	15/42 0.35	6/12 0.50	8/31 0.26	4/21 0.19	13/30 0.43	6/14 0.43	
> 0.80 or extreme skewing	8/44 0.18	—	11/86 0.13	13/44 0.3	4/42 0.10	0/12 0.00	11/31 0.35	12/21 0.57	1/30 0.03	1/14 0.07	
Total skewed	21/44 0.49	2*/20 0.10	37†/86 0.43	21/44 0.49	19/42 0.45	6/12 0.50	19‡/31 0.61	16§/21 0.76	14/30 0.46	7/14 0.50	3/4 0.75

*Only those with discordant patterns, therefore not fair comparison. †This study used methylation PCR + "eye balled" (no densitometry). ‡Using PGK, HPRT, or M27β. §Using skin fibroblasts.
ALD = adrenoleucodystrophy.
IP = incontinentia pigmenti.
PDHEI def = pyruvate dehydrogenase deficiency.

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*²¹ 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).^{29,30} 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.²⁴

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- Revesz T, Schuler D, Goldschmidt B, Elodi S. Christmas disease in one of a pair of monozygotic twin girls, possibly the effect of Lyonisation. *J Med Genet* 1972;9:396-400.
- Tuckerman E, Webb T, Bunday SE. Frequency and replication status of the fragile X, fra(X)(q27-28), in a pair of monozygotic twins of markedly differing intelligence. *J Med Genet* 1985;22:85-91.
- Krueyer H, Mila M, Glover G, Carbonell P, Ballesta F, Estivill X. Fragile X syndrome and the (CGG)n mutation: two families with discordant MZ twins. *Am J Hum Genet* 1994;54:437-42.
- Philip J, Vogelius Andersen CH, Dreyer V, *et al*. Colour vision deficiency in one of two presumably monozygotic twins with secondary amenorrhoea. *Ann Hum Genet* 1969;33:185-95.
- Jorgenson AL, Philip J, Raskind WH, *et al*. Different patterns of X inactivation in MZ twins discordant for red-green colour-vision deficiency. *Am J Hum Genet* 1992;51:291-8.
- Winchester B, Young E, Geddes S, *et al*. Female twin with Hunter disease due to non-random inactivation of the X-chromosome: a consequence of twinning. *Am J Med Genet* 1992;44:834-8.
- Marguery MC, Giordano F, Parant M, *et al*. Fabry's disease: heterozygous form of different expression in two monozygous twin sisters. *Dermatology* 1993;187:9-15.
- Costa T, Greer W, Duckworth-Rysiecki G, Musarella M, Ray P. Monozygotic twins discordant for Aicardi syndrome. *Am J Hum Genet* 1990;47:A53.
- Gomez MR, Engel AG, Dewald G, Peterson HA. Failure of inactivation of Duchenne dystrophy X-chromosome in one of female identical twins. *Neurology* 1977;27:537-41.

- Burn J, Povey S, Boyd Y, *et al*. Duchenne muscular dystrophy in one of monozygotic twin girls. *J Med Genet* 1986;23:494-500.
- Chuthow JG, Hyser CL, Edwards JA, Heffner RR Jr, Czorny JJ. Monozygotic female twin carriers discordant for the clinical manifestations of Duchenne muscular dystrophy. *Neurology* 1987;37:1147-51.
- Pena SDJ, Karpati G, Carpenter S, Fraser FC. The clinical consequences of X-chromosome inactivation: Duchenne muscular dystrophy in one of monozygotic twins. *J Neurol Sci* 1987;79:337-44.
- Richards CS, Watkins SC, Hoffman EP, *et al*. Skewed X-inactivation in a female MZ twin results in Duchenne muscular dystrophy. *Am J Hum Genet* 1990;46:672-81.
- Luspi JR, Garcia CA, Zoghbi HY, Hoffman EP, Fenwick RG. Discordance of muscular dystrophy in monozygotic female twins: evidence supporting asymmetric splitting of the inner cell mass in a manifesting carrier of Duchenne dystrophy. *Am J Med Genet* 1991;40:354-64.
- Zneimer SM, Schneider NR, Richards CS. In situ hybridisation shows direct evidence of skewed X inactivation in one of monozygotic twin females manifesting Duchenne muscular dystrophy. *Am J Med Genet* 1993;45:601-5.
- Nance WD. Do twin Lyons have larger spots? *Am J Hum Genet* 1990;46:646-8.
- James WH. Anomalous X chromosome inactivation: the link between female zygotes, monozygotic twinning, and neural tube defects? *J Med Genet* 1988;25:213-16.
- Boyd Y, Fraser NJ. Methylation patterns at the hypervariable X-chromosome locus DXS255 (M27β); correlation with X-inactivation status. *Genomics* 1990;7:182-7.
- Hendriks RW, Hinds H, Chen ZY, Craig IW. The hypervariable DXS255 locus contains a LINE-1 repetitive element with a CpG island that is extensively methylated only on the active X chromosome. *Genomics* 1992;14:598-603.
- Harris A, Collins J, Vetrie D, Cole C, Bobrow M. X-inactivation as a mechanism of selection against lethal alleles: further investigation of incontinentia pigmenti and X linked lymphoproliferative disease. *J Med Genet* 1992;29:608-14.
- Goodship J, Carter J, Speer A, Burn J. X-inactivation patterns in monozygotic and dizygotic female twins. *Am J Med Genet* (in press).
- Richards CS, Hall JG, Faltermann ML, Nance WE. Skewed X-inactivation in normal monozygotic twin pairs. *Am J Hum Genet* 1990;47:A105.
- Pettigrew AL, Smith JL, Moses R. Phenotypically discordant monozygotic twins - cytogenetic discordance masked by twin-twin transfusion? *Am J Hum Genet* 1990;47:A37.
- Brown RM, Brown GK. X-chromosome inactivation and the diagnosis of X-linked disease in females. *J Med Genet* 1993;30:177-84.
- Tan SS, Williams EA, Tam PPL. X-chromosome inactivation occurs at different times in different tissues of the post-implantation mouse embryo. *Nature Genet* 1993;3:170-4.
- Watkiss E, Webb T, Bunday S. Is skewed X inactivation responsible for symptoms in female carriers for adrenoleucodystrophy? *J Med Genet* 1993;30:651-4.
- Vetrie D, Flinter F, Bobrow M, Harris A. X inactivation patterns in females with Alport's syndrome: a means of selecting against a deleterious gene? *J Med Genet* 1992;29:663-6.
- Bushby KMD, Goodship JA, Nicholson LVB, Johnson MA, Haggerty ID, Gardner-Medwin D. Variability in clinical, genetic and protein abnormalities in manifesting carriers of Duchenne and Becker muscular dystrophy. *Neuromusc Dis* 1993;3:57-64.
- Glass IA, Nicholson LVB, Watkiss E, *et al*. Investigation of a female manifesting Becker muscular dystrophy. *J Med Genet* 1992;29:578-82.
- Robinson DO, Boyd Y, Cockburn D, Collison MN, Craig I, Jacobs PA. The parental origin of de novo X-autosome translocations in females with Duchenne muscular dystrophy, revealed by M27β methylation analysis. *Genet Res* 1990;56:135-40.