X inactivation analysis in a female with hypomelanosis of Ito associated with a balanced X;17 translocation: evidence for functional disomy of Xp

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Abstract
X inactivation analysis was performed on normal and hypopigmented skin samples obtained from a female with hypomelanosis of Ito associated with a balanced whole arm X;17 translocation. Severe skewing of X inactivation resulting in inactivity of the intact X was found in blood and cultures of both types of skin, but analysis of DNA prepared directly from hypopigmented skin showed significant inactivation of the translocated X, inconsistent with the usual mechanism of phenotypic expression in X;autosome translocations. In addition, dual colour FISH analysis using centromere specific probes for chromosomes X and 17 showed that the breakpoints on both chromosomes lie within the alphoid arrays, making interruption of a locus on either chromosome unlikely. While partial variable monosity of loci on chromosome 17p cannot be excluded as contributing to the phenotype in this patient, it is argued that the major likely factor is partial functional disomy of sequences on Xp in cell lineages that have failed to inactivate the intact X chromosome.

(J Med Genet 1996;33:216–220)

Key words: hypomelanosis of Ito; X;autosome translocation; incontinentia pigmenti; functional disomy Xp.

Hypomelanosis of Ito (HI) is a disorder of heterogeneous aetiology. It is characterised by streaks or whorls, or both, of hypopigmentation often associated with CNS abnormalities. Recent evidence suggests that the common feature in many patients with HI is the presence of two or more genetically distinct cell lines.2-4 The description of a number of females with balanced X;autosome translocations and a cytogenetic breakpoint in Xp11, described variously as having HI or incontinentia pigmenti (IP), prompted the suggestion that the locus for IP was at Xp11.5-12 Linkage analysis in familial IP, however, excluded this region and significant evidence now exists for the presence of a locus at Xq28.12-15 In addition, it is now clear that the phenotype described in females with HI associated with X;autosome translocations is not consistent with familial IP. Confusion between the two conditions results partly from the alternative name for HI (incontinentia pigmenti achromians) and the similarity of the skin lesions in the two conditions.16 However, significant differences between the two conditions are evident, including fixed skin abnormalities in HI compared with evolving abnormalities in IP and the absence of dysmorphic features in IP, unlike HI.16

In order to examine the hypothesis that it is the functional status of the translocated X, rather than the site of the X breakpoint, that is significant in producing the phenotype of HI, X inactivation analysis was performed on skin derived from affected (hypopigmented) and unaffected skin in a female with HI associated with a balanced, constitutional whole arm X;17 translocation.

Case report
This female has previously been reported.10,17 Severe psychomotor delay was noted with a developmental level of 8–11 months at the age of 2 years 5 months. She stood alone at this age, but never walked. Dysmorphic features noted then included hypertelorism, a broad, flat nasal bridge, and epicantid folds. At the age of 21, she remains globally severely delayed. She is unable to sit unaided and has no communication. She is dysmorphic with hypertelorism and macrocephaly (95th centile) (fig 1). Her hands are small. Examination of the skin shows whorls and streaks of hypopigmented skin, particularly on the back and lower limbs (fig 2).

Materials and methods
X inactivation analysis by PCR was carried out on DNA extracted from blood lymphocytes, hypopigmented and normal skin, and cell cultures of hypopigmented and normal skin. An
HpaII site which is methylated exclusively on inactive X chromosomes and which is close to a polymorphic site within the human androgen receptor gene (HUMAR) was analysed using the method described by Allen et al. The PCR primers were as described previously: primer 1, 5'GCTGTGAAGGTTGCTGTTC-CTCAT3', primer 2, 5'TCAGAATCTGGTCCAGAGCCGTG3'.

DNA (1 μg) was predigested for 16 hours at 37°C with HpaII in a volume of 20 μl containing 10 mmol/l Tris HCl, pH 8.3, 50 mmol/l KCl, 2 mmol/l MgCl2, 230 μmol/l each dNTP, 0.8 μmol/l each primer, and 0.5 units of HpaII. Controls for each DNA sample were treated in exactly the same way except that no restriction enzyme was added.

Two microlitres of the HpaII digest were amplified by PCR using the HUMAR primers described above. Primer 2 was end labelled using T4 polynucleotide kinase and gamma 32P ATP.

The reaction contained 10 mmol/l Tris HCl, pH 8.3, 50 mmol/l KCl, 2 mmol/l MgCl2, 230 μmol/l each dNTP, 0.8 μmol/l each primer, and 0.5 units of Taq polymerase in a volume of 50 μl.

An initial denaturation step of 96°C for seven minutes was followed by 28 cycles of 95°C for one minute, 60°C for one minute, and 72°C for two minutes with a final extension step of 72°C for seven minutes.

PCR products (5 μl) were added to an equal volume of gel electrophoresis buffer (95% formamide, 20 mmol/l EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol), heated at 100°C for five minutes, and loaded onto a denaturing 6% 39:1 acrylamide/bisacrylamide sequencing gel containing 6 mol/l urea in Tris borate buffer. Bands were visualised and band intensities measured using a Fuji phosphor-imager.

Fluorescence in situ hybridisation (FISH) was performed using a standard protocol with biotin labelled X centromere probe (DXZ2) and digoxigenin labelled 17 centromere alhpoid repeat (D17Z1). Following stringent washing, the sites of hybridisation were visualised using dual colour fluorescence reporter molecules (Texas Red for biotin and FITC for digoxigenin labelled probes, respectively). The signals were captured using a cooled CCD camera and enhanced with Smartcapture software (Digital Scientific, Cambridge).

For late labelling studies, fibroblasts derived from both skin biopsies were cultured with the addition of BrdU for the last eight hours before harvest. The metaphases obtained were stained with acridine orange (10 μg/ml) and viewed under epifluorescence.

### Results

The results of X inactivation analysis by PCR are shown in fig 3 and table 1. DNA extracted from blood and from cultured normal and hypopigmented skin showed a severe skewing of X inactivation indicating that the translocated X was active in almost 100% of cells, as expected. However, analysis of DNA from uncultured hypopigmented skin showed a more

### Table 1

<table>
<thead>
<tr>
<th>Hypopigmented skin</th>
<th>Normal</th>
<th>Blood</th>
</tr>
</thead>
<tbody>
<tr>
<td>Direct Derived X</td>
<td>59:2</td>
<td>98:15</td>
</tr>
<tr>
<td>Derived X</td>
<td>40:8</td>
<td>1:85</td>
</tr>
<tr>
<td>Cultured Derived X</td>
<td>98:2</td>
<td>96:8</td>
</tr>
<tr>
<td>Intact X</td>
<td>1:8</td>
<td>3:2</td>
</tr>
</tbody>
</table>
balanced pattern of X inactivation, indicating that the intact X was active in approximately 40% of cells.

BrdU analysis of cells cultured from blood, hypopigmented skin, and normal skin showed in all cases the normal X to be almost exclusively inactive (table 2).

Dual colour FISH showed that both translocation products had compound centromeres, that is, were composed of both red (X centromere) and green (17 centromere) signals, indicating that the breakpoint in both translocation chromosomes had occurred within the alphoid arrays (fig 4).

Fig 3 also showed the active translocated X to be paternal in origin. This is consistent with the 12 other cases of balanced de novo X;autosome translocations which have been studied, all of which are paternal in origin.20

Discussion
The discovery of balanced X;autosome translocations in females manifesting characteristic X linked phenotypes normally seen only in males has been of enormous benefit in the

localisation and eventual isolation of the genes responsible for a number of disorders, including Duchenne muscular dystrophy, Menkes disease, and Lowe’s syndrome.24-26 In females possessing such balanced X;autosome translocations, it is usually found that the translocated X is active in the great majority of, if not all, cells (although analysis is usually only performed on peripheral blood leucocytes). While X inactivation is thought to occur randomly initially, cells that have inactivated the translocated X may be at a selective disadvantage, either because of spreading of X inactivation into the translocated autosome or, more likely, because of the functional disomy of sequences on the X distal to the site of the X breakpoint.27 An abnormal phenotype results because X inactivation is severely skewed, so that the intact X is always inactive (and thus so is the relevant locus) while the active (trans-
located) X has a non-functional locus owing to direct interruption.

In the case of the X;17 translocation described here, two findings make it unlikely that the classical mechanism is relevant. (1) Dual colour FISH analysis shows the breakpoints to be centromeric on each chromosome, making direct interruption of a gene highly unlikely. (2) While the pattern of X inactivation is as expected in most samples, it differs significantly in affected, uncultured skin. It is likely that cells that have an inactive derived X are selected against during culture and thus their presence can only be deduced by direct analysis of affected skin which has not been cultured (that such selection takes place at all may be an indication of how abnormal such "near lethal" cells are).

It is noteworthy that X inactivation analysis of uncultured tissue would not have been possible before the advent of molecular techniques, such as used in this study. Thus, it is likely that the unusual pattern of X inactivation found in hypopigmented skin from our patient would not have been detected in the past because of the reliance of techniques on cell culture. It is clear, however, that despite eradication of these cells in culture, they are tolerated in vivo, although the reasons for this are unclear. There are likely to be two consequences of the presence of cells harbouring an inactive derived X chromosome in the affected tissue, namely partial functional disomy of Xp and partial (variable) functional monosomy of 17p (fig 5, bottom).

Much evidence now exists that the presence of functional disomy of sequences on the X, albeit in only a proportion of cells, can confer a severe phenotype in some females. Females with ring X chromosomes are likely to have a severe phenotype if the ring X is unable to inactivate. Some of the females have been found to possess a pterygium dysplasia reminiscent of HI, in addition to CNS abnormalities and dysmorphic features. The evidence provided here points to a correlation between the X inactivation status of the translocated X, and the presence of hypopigmented skin in a female with HI associated with a balanced, whole arm constitutional X;17 translocation. Cells that have inactivated the derived X are likely to have functional disomy of sequences of the X, distal to the breakpoint at Xcen, and variable functional monosity of sequences distal to the centromeric breakpoint on chromosome 17. While it may be argued that the autosomal monosomy may be contributing to the phenotype, it is unlikely to be the main factor as the autosomes involved in other females described with HI in association with a balanced X-autosome translocation are usually different. The presence of functional disomy of Xp in even a small proportion of cells is likely to give rise to a severe phenotype, particularly if the CNS is involved. HI in this female may simply be the manifestation of the presence of a "near lethal" cell line in the skin. Such "near lethality" may result from a number of chromosomal (functional) aneuploidies, of which disomy of Xp is only one example.

We are grateful to Annette Cockwell, Andrew Fisher, and Carolyn Campbell for their help with the analysis of the translocation and the pattern of X inactivation as deduced from BrdU incorporation. We are also grateful to Dr Peter Tarnopenny for his help.


30 De Grouchy J, Turleau C, Doussau DBM, Maroteaux P, Thibaud D. Incontinentia pigmenti (IP) and r(X).
