European Journal of Human Genetics (2008) 16, 897–905

www.nature.com/ejhg

ARTICLE

Molecular and cytogenetic analysis of the spreading of X inactivation in a girl with microcephaly, mild dysmorphic features and t(X;5)(q22.1;q31.1)

Roberto Giorda*[,1,7], M Clara Bonaglia[2,7], Greta Milani[1], Anna Baroncini[3], Francesca Spada[3], Silvana Beri[1], Giorgia Menozzi[4], Marianna Rusconi[1] and Orsetta Zuffardi[5,6]

[1]*Molecular Biology Laboratory, 'E. Medea' Scientific Institute, Bosisio Parini, Lecco, Italy;* [2]*Cytogenetics Laboratory, 'E. Medea' Scientific Institute, Bosisio Parini, Lecco, Italy;* [3]*UO Genetica Medica, AUSL Imola, Imola, Italy;* [4]*Bioinformatic Laboratory, 'E. Medea' Scientific Institute, Bosisio Parini, Lecco, Italy;* [5]*Biologia Generale e Genetica Medica, Università di Pavia, Pavia, Italy;* [6]*IRCCS Policlinico San Matteo, Pavia, Italy*

X chromosome inactivation involves initiation, propagation, and maintenance of gene inactivation. Studies of replication pattern and timing in X;autosome translocations have suggested that X inactivation may spread to autosomal DNA. To examine this phenomenon at the molecular level, we have tested the transcriptional activity of a number of chromosome 5 loci in a female subject with microcephaly, mild dysmorphic features and 46,X,der(X)t(X;5)(q22.1;q31.1) karyotype. RT-PCR analysis of 20 transcribed sequences spanning 5q31.1-qter revealed that nine of them were not expressed in somatic cell hybrid clones carrying the translocated chromosome. However, eight genes were expressed and therefore escaped inactivation. This direct expression test demonstrates that spreading of inactivation from the X chromosome to the adjoining autosomal DNA was incomplete and 'patchy'. Inactivation was associated in most instances to methylation of the CpG sequences in genes containing CpG islands, but was also present in CpG islandless genes. These results agree with those obtained for other X;autosome translocations and demonstrate that autosomes are partially resistant to *Xist*-mediated spreading and/or maintenance of inactivation. Repeat distribution analysis does not suggest an association between L1 and LINE repeat density on chromosome 5 and gene inactivation. The expression data may also explain why the proband manifests an attenuated clinical phenotype compared to subjects with partial chromosome 5 trisomy.
European Journal of Human Genetics (2008) **16,** 897–905; doi:10.1038/ejhg.2008.28; published online 27 February 2008

Keywords: unbalanced translocation; chromosome X inactivation; gene expression; spreading of inactivation

Introduction

X-chromosome inactivation is a mechanism of compensation for the gene dosage differences that exist between the sexes. It randomly silences the majority of genes on one X chromosome in somatic cells of female mammals. The inactive status is stable and maintained through subsequent cell divisions. The mechanism by which X inactivation occurs is still not entirely clear. In mammals, it is regulated by a single *cis*-acting locus, the X inactivation centre (Xic). The Xic transcribes a non-coding RNA, the X inactive-specific transcript (*Xist*), which associates with the chromosome in *cis* and converts the chromosome to a silent heterochromatic configuration.[1–5] Epigenetic changes associated with inactive X chromosomes include

*Correspondence: Dr R Giorda, Molecular Biology Laboratory, 'E. Medea' Scientific Institute, via don Monza 20, Bosisio Parini, Lecco 23842, Italy.
Tel: +39 031 877911; Fax: +39 031 877499;
E-mail: roberto.giorda@bp.lnf.it
[7]These first two authors should be regarded as joint first authors.
Received 4 October 2007; revised 11 January 2008; accepted 17 January 2008; published online 27 February 2008

hypermethylation of CpG islands, covalent modification of core histone tails, and incorporation of variant histones.[6]

When the Xic is translocated onto autosomes, spreading of the silent chromatin structure into autosomal chromatin can occur,[7] but is frequently attenuated and incomplete.[8–10] Initial observations in X;autosome unbalanced translocations have noted the inactivation of single autosomal genes, but only two studies[11,12] have directly measured the spread of X inactivation through autosomal DNA by gene expression analysis. White *et al*[11] found that the spread of inactivation in an X;4 translocation was discontinuous, while Sharp *et al*[12] showed in five X;autosome translocations that spread of gene silencing occurred in either a continuous or discontinuous fashion in different cases.

We have used gene expression and CpG island methylation analysis in somatic cell hybrid clones to assess the inactivation status of several chromosome 5 genes translocated onto the X chromosome in a girl with microcephaly, developmental delay and subtle dysmorphic features carrying an unbalanced 46, X, der(X)t(X;5)(q;q31) karyotype.

Materials and methods

Case report

The female proband was the third child of healthy unrelated Caucasian parents, ages 37 (mother) and 41 (father) at the time of her birth. Her older brother and sister were healthy.

The family history is unremarkable. No drug, infection or radiation exposure during pregnancy is reported. The pregnancy was complicated by fetal chronic distress during the third trimester. Fetal bradycardia and cord prolapse in the presence of intact membranes necessitated an emergency C-section at 39 weeks. At birth, she weighed 2410 g (<3rd centile), was 47 cm long (3rd centile) and her head circumference was 31.5 cm (<3rd centile). APGAR scores were 5/7/8 at 1, 5 and 10 min, respectively. In the neonatal period she was diagnosed with a small muscular ventricular septal defect (VSD), which spontaneously closed within the first year of life. At 20 months, skull X-rays confirmed microcephaly and did not reveal premature fusion of the cranial sutures. At 2 1/2 years, a CT scan of the head showed no structural anomalies of the brain, normal corpus callosum, ventricles and basilar cisterns. At 5 1/2 years, a cerebral MRI was normal.

Audiological assessment at 4 and 6 years of age showed mild bilateral conduction hearing loss (speech reception threshold of 30 dB) attributed to recurrent acute otitis media. Ophthalmologic evaluation showed hypermetropic astigmatism OS > OD and left esophoria.

Her psychomotor development was moderately delayed, and in particular she displayed very short attention span and impaired fine motor skills. From the time of starting school she had learning difficulties and low tolerance frustration.

On examination at age 7 1/2 years, she was 130 cm tall (90th centile), weighed 36 Kg (>97th centile) with a head circumference of 48 cm (<3rd centile). She had brachycephaly (cephalic index = 85.2%), upslanting palpebral fissures, bilateral epicanthic fold, hypertelorism, small and low-set ears (total ear length 4.7 cm, <3rd centile), high nasal bridge, thin lips, microstomia and short neck. Her hands were of normal length, but with tapering fingers, brachydactyly of the fifth fingers and hypoplasic creases. Mild genu valgum was present and all joints were mildly stiff.

At the age of 10 years, she developed polyarticular juvenile arthritis, mainly involving hand and knee joints. Disease onset was insidious with morning stiffness and arthralgia during the day. Rheumatoid factor was negative and immunity-autoimmunity markers were within the normal range.

At last follow-up visit, at 11 years, she was 151 cm tall (90th–97th centile), weighed 56 Kg (>97° centile) and had a head circumference of 50 cm (3rd centile). Fat distribution was predominantly truncal. All standard laboratory parameters were within the normal range and, despite an extensive clinical and ultrasound examination, no further anomalies were found. Karyotype analysis revealed an unbalanced (X;5) translocation.

Cytogenetic studies and Array-CGH

Chromosome analysis was performed on the proband's and parents' blood, using standard high-resolution techniques.[13] The TelVysion kit with telomere-specific probes was used according to the supplier's instructions (Vysis Inc., Downers Grove, IL, USA).

Molecular karyotyping was performed through Array-CGH with Agilent ~100 Kb oligo-chip (Agilent Technologies, Palo Alto, CA, USA; www.chem.agilent.com). The Agilent kit platform is a 60-mer oligonucleotide-based microarray that allows genome-wide survey and molecular profiling of genomic aberrations with a resolution of ~100 kb (kit 44B). DNAs (500 ng) of the patient and controls of the same sex (Promega Co., Fitchburg, WI, USA) were double-digested with *Rsa*I and *Alu*I for 2 h at 37°C. After column purification, 2 μg of each digested sample were labelled by random priming (Invitrogen, Carlsbad, CA, USA) for 2 h using Cy5-dUTP for the patient's/ parents' DNA and Cy3-dUTP for the control DNA. Labelled products were column purified. After probes denaturation and pre-annealing with 50 μg of Cot-1 DNA, hybridisation was performed at 65°C with rotation for 40 h. After two washing steps, the array was analysed through the Agilent scanner and Feature Extraction software (v8.1). A graphical overview was obtained using the CGH analytics software (v3.4.4).

Somatic cell hybrid generation and molecular analysis

Somatic cell hybrid clones were generated fusing the HPRT-negative RJK88 Chinese hamster cell line with lymphoblastoid cell line from the patient.[14] Clones were tested for the presence of der(X) or chromosome 5 by PCR with polymorphic markers.

Genomic DNA was extracted from the proband's lymphoblastoid cell line and her parent's blood using standard protocols. Somatic cell hybrid clones' DNA was extracted with DNAzol (MRC, Inc, Cincinnati, OH, USA). Genotyping of polymorphic loci was performed by amplification with primers labelled with fluorescent probes (ABI 5-Fam, Hex and Tet) followed by analysis on an ABI 310 Genetic Analyzer (Applied Biosystems, Monza, Italy). Non-polymorphic loci were assayed by electrophoresis on agarose gels.

The UCSC Genome Browser (hg17 and hg18 assemblies; http://genome.ucsc.edu/) maps and sequence were used as references.

Sequencing reactions were performed with a Big Dye Terminator Cycle Sequencing kit (Applied Biosystems) and run on an ABI Prism 3130xl Genetic Analyzer.

RT-PCR analysis

Total RNA was extracted from all tissues and cell lines with Trizol (Invitrogen) following manufacturer's protocols; all cDNA synthesis were performed with Ready-To-Go You-Prime First strand beads (GE Healthcare Europe, Milano, Italy) and random hexamers. The cDNAs were amplified with PCR using primers for the individual loci to be tested. Primer sequences are available from the authors on request. Primer pairs that amplified a fragment from genomic DNA or RJK88 cDNA were excluded. Only loci expressed in the proband's EBV line were used. RT-PCR was performed in 25 μl reactions, using JumpStart Red ACCUTaq LA DNA polymerase (Sigma-Aldrich, St. Louis, MO, USA) and the following protocol: 1 min at 96°C; 30 cycles of 30 s at 94°C/30 s at 58°C/2 min at 68°C; 5 min at 68°C final elongation time; PCR products were analysed on 1.5% agarose TAE gels. *G3PDH* amplification primers and protocol are from Clontech, Mountain View, CA, USA.

CpG island methylation and chromosome X inactivation analysis

We analyzed CpG methylation in six promoter-associated CpG islands. Bisulphite treatment was carried out as described.[15] Methylation levels were tested by bisulphite restriction mapping of the amplified fragments with the *Taq*I restriction enzyme; after the bisulphite reaction, the unmethylated DNA remains intact following *Taq*I digestion, whereas the methylated DNA is cleaved. X chromosome inactivation was analyzed using a polymorphic CAG repeat in the androgen-receptor (AR) gene as described.[16]

Repeat distribution analysis

We retrieved all the repeats and genes data for chromosome 5 from the UCSC genome browser (http://genome.ucsc.edu/) using the latest assembly (hg18). This resulted in a total of 209531 repeats features. The frequencies of Alu, MIR, LINE and L1 elements have been calculated starting from 55 MB in nonoverlapping windows of 100 Kb. The data on CTCF binding sites were downloaded from the authors' website (http://licr-renlab.ucsd.edu/download.html); since genome coordinates were expressed in relation to the hg17 assembly, we used the liftOver software (http://genome.brc.mcw.edu/cgi-bin/hgliftOver) to convert the positions to the latest assembly. All analyses have been performed using R (http://www.R-project.org).[17]

Results

Karyotype analysis in the proband showed a derivative X chromosome containing extra material transposed at the level of Xq22. 1 (Figure 1a). Further analysis with FISH subtelomeric probes demonstrated the Xq deletion (Figure 1c) and the presence of additional chromosome 5q material on the der(X) chromosome (Figure 1d). The rearrangement was rapidly characterised by Array-CGH analysis: the chromosome Xq deletion covers the distal 52.316 Mb and the breakpoint is located between oligomers at 101 719 Mb (still present) and 101 808 Mb (first deleted) (Figure 2a); the duplicated 5q region encompasses the terminal 48 843 Mb with the breakpoint flanked by oligomers at 133.774 Mb (not duplicated) and 133.899 Mb (first duplicated) (Figure 2b).

The final karyotype interpretation was 46, X, der(X)t(X;5)(q22.1;q31.1).

We created somatic cell hybrids and screened 56 clones with chromosome 5 and X polymorphic markers. We found two clones containing a maternal chromosome 5, one with a paternal chromosome 5, two with der(X), two containing both chromosome 5 and der(X). Since hybrid clones selection is based on the complementation of the HPRT-negative rodent cell line with the human HPRT gene on the active X chromosome, all clones also contained the normal X chromosome.

Typing with polymorphic chromosomes 5 (Table 1) and X (Table 2) markers demonstrated that the der(X) chromosome contained maternal chromosome X and paternal chromosome 5 material. The normal paternal chromosome 5 and the portion of chromosome 5 on the der(X) came from the same homologue. Chromosome X inactivation analysis revealed that, as expected, the proband's der(X) chromosome was selectively inactivated (Figure 3a).

By PCR amplification, we narrowed the chromosome 5 breakpoint location to a 7.5 Kb repeat region (chr5:133 832 871 – 133 840 562). This region contains a

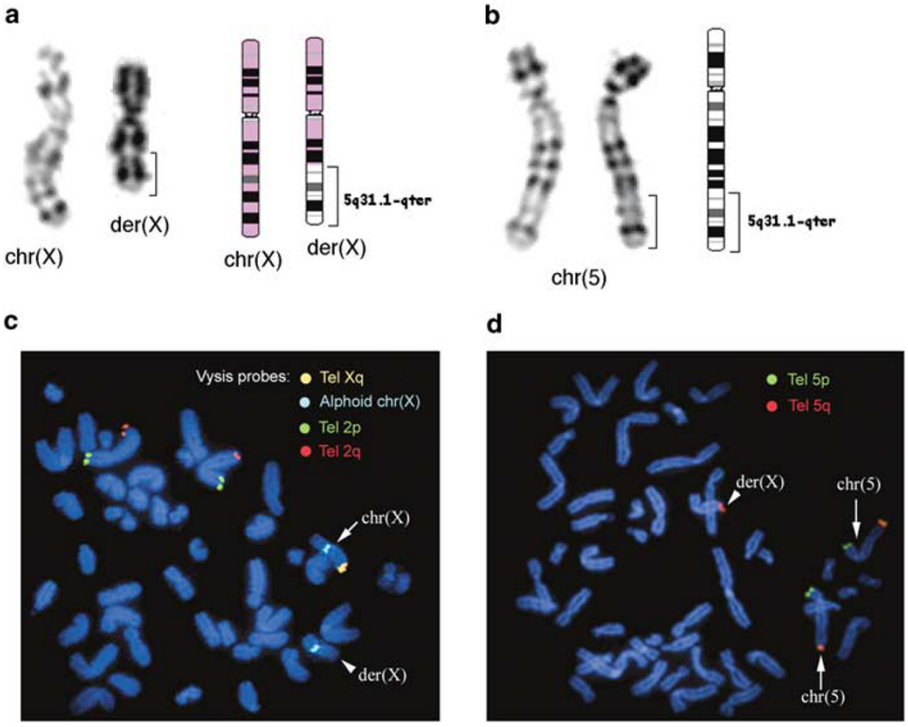

Figure 1 (**a**) *(left)* Cut out of normal chromosome X aligned with its translocated der (X) homologue in G-banding at a resolution of $\geq$550 bands; (*right*), Corresponding high-resolution G-banding ideogram of chromosome X (*pink*) and der (X) (*pink, with black/white portion*). (**b**) (*left*) Cut out of normal chromosome 5 homologues in G-banding, at a resolution of $\geq$550 bands; (*right*), corresponding high-resolution G-banding ideogram of chromosome 5 (*black/white*). Square brackets indicate the translocated portion containing sub-bands 5q31.1-qter. No Xq material is visible on other chromosomes, when examined by cytogenetics or FISH with an X-painting library. (**c**, **d**) FISH results with the Xq and 5q subtelomeric-specific probes (Vysis). (**c**) FISH analysis with the subtelomeric Xq probe (Tel Xq, Spectrum yellow TelVysion, Vysis) shows signals only on one chromosome X (arrow), while the der (X) does not show any hybridisation signal (arrowhead). The probe mix also contains a second set of differentially labelled/coloured subtelomeric probes, which were localised to different chromosomes: tel(2p) (spectrum green), tel(2q) (spectrum orange) and alphoid chr X (spectrum aqua). These probes served as internal controls for hybridisation efficiency. (**d**) FISH analysis with the subtelomeric 5q probe (Tel 5q, spectrum orange, TelVysion, Vysis) shows signals on the two normal chromosomes 5 (arrow) and on the der(Xq) (arrowhead). The probe mix also contains a second differentially labelled 5p subtelomeric probe (Tel 5p, spectrum green).

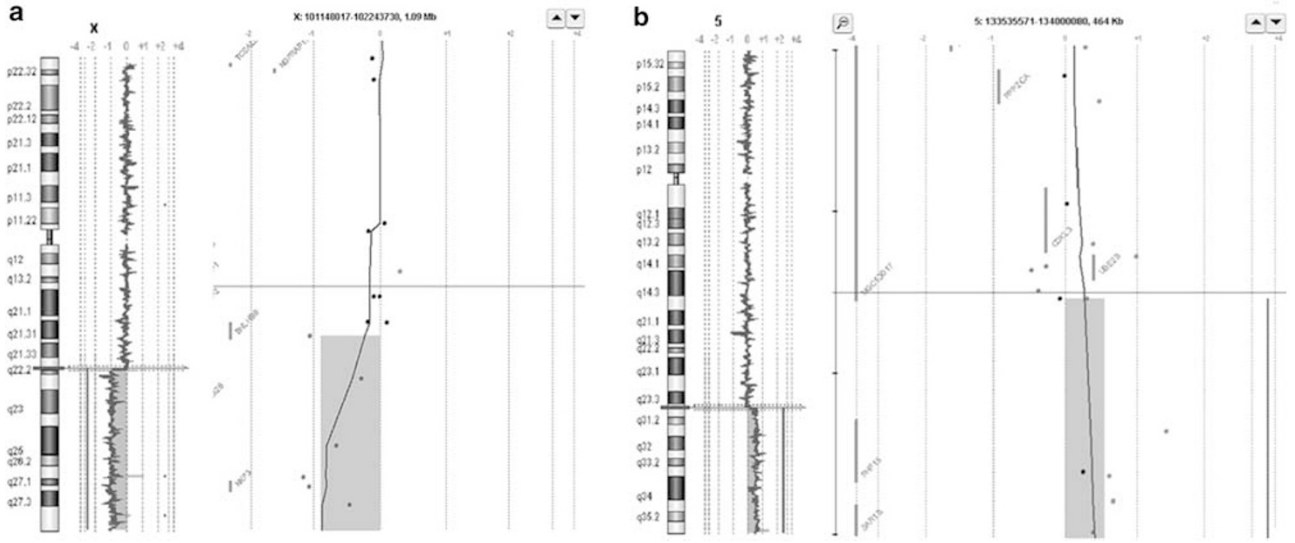

Figure 2 Array-CGH profile of chromosome X (**a**), showing the Xq22.1-Xqter monosomy and chromosome 5 (**b**), showing 5q31.1-5qter trisomy; on the right, detail of the region containing the breakpoint on chromosome Xq22.1 and 5q31.1, respectively.

Table 1 Typing with chromosome X polymorphic markers

Marker	*Position (Kb)*	*Proband*	*Mother*	*Father*	*Chr X*	*Chr X/der(X)*
DXS 1060	5.420	237/243	237/245	243	243	237/243
DXS 1226	22.857	349/351	349/351	351	351	349/351
DXS 1202	26.381	210/212	210/214	212	212	210/212
DXS 1214	31.171	282/284	280/282	284	284	282/284
DXS 1068	38.793	244	244/250	244	244	244
DXS 993	41.032	261/277	277	261	261	261/277
DXS 1055	46.311	77/79	77/79	77	77	77/79
DXS 991	55.536	319/327	319/323	327	327	319/327
DXS 986	79.268	157/167	157	167	167	157/167
DXS 990	92.887	118/122	118/122	118	118	118/122
DXS 1106	102.618	108	111	108	108	108
DXS 1101	119.720	200	202/208	200	200	200
DXS 1047	128.903	156	154/164	156	156	156
DXS 1227	140.630	74	78/82	74	74	74

Molecular typing was performed on genomic DNA from the proband, her parents, a somatic cell hybrid clone containing the normal chromosome X (Chr X), and a clone containing both the normal X and the der(X) chromosomes (Chr X/der(X)).

Table 2 Typing with chromosome 5 polymorphic markers

Marker	*Position (Kb)*	*Proband*	*Mother*	*Father*	*Chr 5-1*	*Chr 5-2*	*Chr der(X)*
D5S 2002	132.404	169/175	173/175	169/173	175	169	—
D5S 2117	133.065	218/226	224/226	218/228	226	218	—
D5S393	135.729	163/165*	163	165/173	163	165	165
D5S399	135.991	111/123*	111	121/123	111	123	123

Molecular typing was performed on genomic DNA from the proband, her parents, two somatic cell hybrid clones containing the maternal and paternal normal chromosomes 5 (Chr 5-1 and 5-2), and a clone containing the der(X) chromosome (Chr der(X)).
Duplicated alleles are indicated by an asterisk (*).

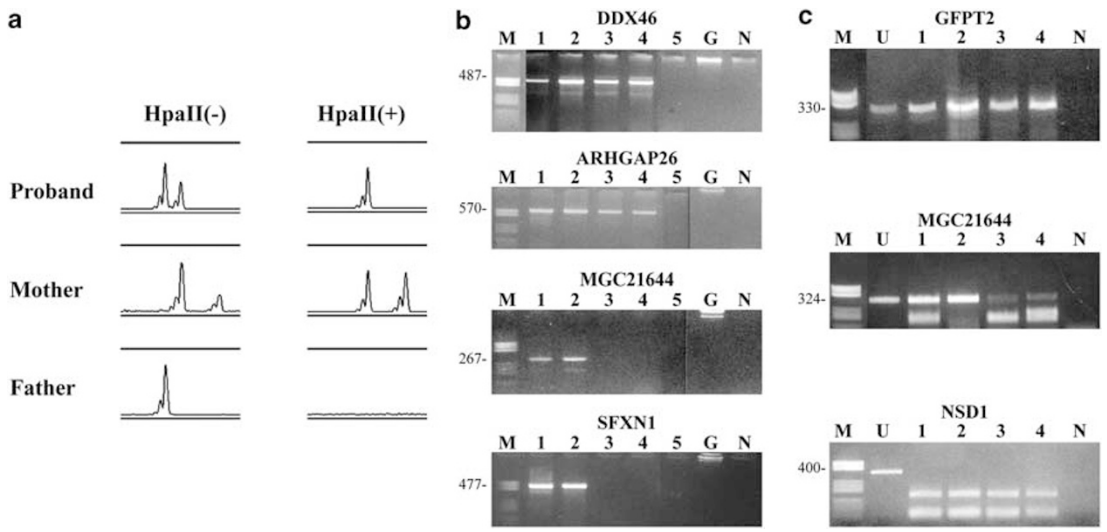

Figure 3 (**a**) X chromosome inactivation analysis of the proband and her parents. *Hpa*II(−), before digestion with *Hpa*II; *Hpa*II(+), after digestion with *Hpa*II for 5 h at 37°C. Before *Hpa*II digestion, all AR alleles are amplified. Following *Hpa*II digestion and amplification, PCR products are obtained only from the AR allele on the inactive X chromosome. (**b**) Expression analysis of four genes on chromosome 5q. (1) cDNA from the proband's EBV line; (2) cDNA from chromosome 5-containing somatic cell hybrid clone; (3 and 4) cDNA from chromosome der(X)-containing somatic cell hybrid clones; cDNA from negative control somatic cell hybrid clone; G, genomic DNA; N, water control; M, molecular weight marker V, GE Healthcare. The sizes of all amplified fragments are shown. (**c**) CpG methylation analysis of three chromosome 5q genes. U, Na Bisulfite-modified genomic DNA, amplified, uncut; (1) proband's bisulfite-modified DNA, TaqI cut; (2) chromosome 5-containing somatic cell hybrid clone, bisulfite-modified DNA, TaqI cut; (3 and 4) chromosome der(X)-containing somatic cell hybrid clones, bisulfite-modified DNA, TaqI cut; N, water control; molecular weight marker V, GE Healthcare. The sizes of all amplified uncut fragments are shown.

Table 3 Analysis of allele-specific expression of chromosome 5q22-qtel genes

Gene	*Position (Kb)*	*Orient.*	*CpG*	*EBV line*	*Chr 5*	*Der(X) cl. 1*	*Der(X) cl. 2*	*Neg.*	*DNA*	*Status*
DDX46	134122–134194	+	Y	+	+	+	+	–	–	Active
SPOCK1	136338–136862	–	Y	+	+	–	–	–	–	Inactive
SIL1	138310–138561	–	N	+	+	+	+	–	–	Active
ANKHD1	139761–139832	+	Y	Traces	+	+	+	–	–	Active
HDAC3	140980–140996	–	Y	Traces	Traces	Traces	–	–	–	?
ARHGAP26	142130–142586	+	Y	+	+	+	+	–	–	Active
SMAP-5	143517–143530	–	Y	+	+	–	–	–	–	Inactive
MGC21644	145118–145195	–	Y	+	+	–	–	–	–	Inactive
SPARC	151021–151046	–	N	+	+	+	–	Traces	–	Active
GEMIN5	154247–154297	–	Y	+	+	–	–	–	–	Inactive
EBF	158058–158459	–	Y	+	+	–	–	–	–	Inactive
TTC1	159368–159425	+	N	+	+	–	–	Traces	–	Inactive
WWC1	167651–167829	+	Y	Traces	Traces	–	–	–	–	Inactive
LCP2	169607–169657	–	N	+	+	–	–	–	–	Inactive
ERGIC1	172193–172312	+	Y	+	+	+	–	–	–	?
CPEB4	173248–173319	+	Y	+	+	–	+	–	–	?
SFXN1	174838–174888	+	Y	+	+	–	–	–	–	Inactive
NSD1	176494–176655	+	Y	+	+	+	+	–	–	Active
AK094065	177490–177508	+	Y	+	+	+	+	–	–	Active
GFPT2	179660–179712	–	Y	+	+	+	+	–	–	Active

Gene expression was analysed by RT-PCR on RNA from a proband-derived EBV line, a somatic cell hybrid clone containing chromosome 5 (Chr 5), two clones carrying the der(X) chromosome (Der(X)cl. 1 and 2), and a clone carrying neither chromosome 5 or der(X). None of the loci we tested amplifies a band of similar size from human genomic DNA (DNA) or RNA from the hamster cell line (not shown).

Table 4 Promoter CpG island methylation analysis of chromosome 5q22-qtel genes

Locus	*Position (Kb)*	*Der(X) inactivation*	*Chr 5*	*Der(X)*	*Proband EBV*	*Control EBV*
MGC21644	145118–145195	Inactive	U	M	U/M	U
GEMIN5	154247–154297	Inactive	U	M	U/M	U
EBF	158058–158459	Inactive	U	U and M	U/M	U/M
WWC1	167651–167829	Inactive	U	M	U/M	U
NSD1	176494–176655	Active	M	M	M	M
GFPT2	179660–179712	Active	U	U	U	U

We analysed DNA from the proband's EBV line, the somatic cell hybrid clone containing chromosome 5 (Chr 5), the two clones carrying der(X) (Der(X)), and a control EBV line. The *EBF* gene was completely methylated in one of the der(X) clones and completely unmethylated in the other (U and M); the same gene was preferentially, but not completely, unmethylated in both proband and control EBV lines.

mix of SINE, LINE, LTRs and simple repeats. We did not refine the chromosome X breakpoint location further.

To test the expression of genes located in the portion of chromosome 5 translocated to the der(X), we selected 20 transcripts regularly spaced at 2 Mb intervals between 5q31 and 5qter (see Table 3) and analyzed them by RT-PCR on RNA from the original EBV line, a somatic cell hybrid clone containing chromosome 5, two clones carrying the der(X), and appropriate controls (see Figure 3b for examples).

Eight of the 20 genes tested are expressed in both chromosome 5 and der(X)-containing clones, thus escaping inactivation; nine genes are selectively expressed in the chromosome 5-containing hybrid and inactive on the der(X); three genes did not give a clear-cut result (Table 3).

Active and inactive genes are apparently randomly located on the chromosome, although we found a majority of active genes between 134 000 and 142 000 Kb (5q31), followed by a majority of inactive genes between 143 500 and 175 000 Kb (5q32-5q35.2), and again by active genes between 176 000 Kb and the telomere (5q35.3); five active and three inactive genes are in the + orientation; six active and seven inactive genes have a promoter CpG island.

To analyze the association between inactivation and promoter CpG island methylation status, we tested four inactive and two active genes by bisulphite restriction mapping of the EBV line, the somatic cell hybrid clone containing chromosome 5, the two clones carrying der(X), and appropriate controls (Figure 3c for examples, and Table 4). The methylation status of all genes was also assayed in a number of control EBV lines. In the two genes that did not undergo inactivation, CpG island methylation did not change between chromosomes 5 and der(X): *GFPT2* was always unmethylated, while *NSD1* was methylated. Promoter CpG islands of all four inactivated genes were unmethylated in chromosome 5 and methylated in der(X)-containing clones. The results were clear cut for

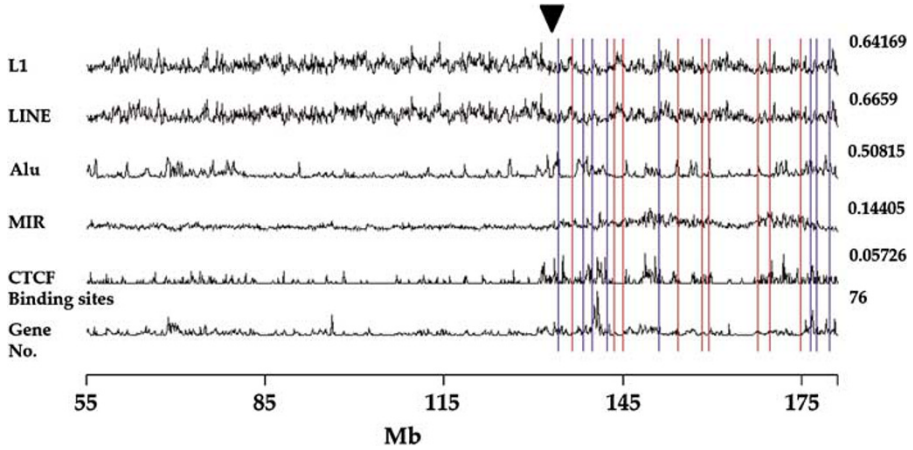

Figure 4 Plot of the long arm of chromosome 5 (chr5:55 000 000–180 857 866) showing the frequency values for repeat elements calculated for 100 KB non-overlapping windows. The CTCF site density profiles and of the number of genes found in each window are also shown; the maximum is reached near 140 Mb. The positions of the eight genes that escaped inactivation are represented with vertical blue lines, those of the nine inactivated genes by red lines. Horizontal light grey lines indicate the mean values for the region examined. On the right side of the plot, we have also reported the maximum value reached by each profile. The breakpoint's position is indicated by a black arrowhead.

GEMIN5 and *MGC21644*, while for *EBF* and *WWC1* only one of the der(X) clones was methylated, although the genes were not expressed in either of them. For *WWC1* the chromosome 5 clone was also partially methylated.

Since L1 elements have been suggested as candidates for the way stations boosting the inactivation signal,[18,19] we sought to determine L1 density in the translocated portion of chromosome 5. We calculated the percentage of L1, MIR, Alu, LINE content and gene density along the long arm of human chromosome 5. It has recently been shown that CTCF binds at the boundary of domains escaping X-chromosome inactivation on the X chromosome.[20] Consequently, using available genome-wide CTCF-binding mapping data,[21] we assayed CTCF binding sites density and association to active and inactive chromosome 5 genes. The data are shown in Figure 4, along with the position of the genes we tested along the chromosome. The strong correlation between gene density and CTCF binding sites frequency mentioned by Kim *et al*[21] was verified. No difference for any of the analyzed elements was detected performing a Wilcoxon test on the frequencies of the different repeats and CTCF-binding sites in the two datasets of activated and inactivated genes. The same results hold true when considering a 1 Kb frequency window and extending the analysis on both sides of the transcribed regions.

Discussion

Origin of the rearrangement

The case we present concerns a female subject with 46,X,der(X)t(X;5)(q22.1;q31) karyotype, presenting *de novo* deletion of the distal 50 Mb of one X chromosome (Xq22.1-Xqter) and duplication of a 5q31-qter segment of about 50 Mb. We can deduce that the rearrangement originated from a non-disjunction of chromosome 5 during paternal meiosis II, since the normal paternal chromosome 5 and the portion of chromosome 5 translocated on the der(X) came from the same homologue. The translocation onto the X chromosome was postzygotic and allowed rescue of the trisomy. Given the absence of mosaicism, the translocation probably happened very early in development, probably during the first postzygotic divisions. Two additional cases of postzygotic X;autosome translocations have been reported, one involving paternal 14q,[22] the other maternal 17q22.[23]

The phenotype

In most cases, the clinical phenotype in subjects with trisomy derived from an unbalanced X;autosome translocation is milder than in subjects with other kinds of trisomy. This is due to partial inactivation of the autosomal genes, and in most cases a good correlation can be observed between the attenuation of the clinical phenotype associated to the partial trisomy and the degree of gene silencing.

The 5q35-qter duplication syndrome, to which the Hunter–Mc Alpine syndrome[24] is assimilated, presents a phenotype of mental and growth retardation, microcephaly, strabismus, prominent nasal bridge, long philtrum, low-set dysplastic ears, downslanting palpebrae, epicanthus, hypertelorism, brachydactyly, genital defects, pulmonary hypertension, cardiac defects, congenital hip dysplasia, dental caries and eczema.[25–27]

In the only other cases of 5q trisomy derived from an unbalanced X;5 translocation in the literature,[27,28] the

clinical picture had most of the typical features of trisomy 5. In the second case, where the breakpoint was on chromosome 5q33, the subject showed a milder phenotype presenting low stature, prominent nasal bridge, thin upper lip, dental caries, mild cardiac defects. The patient we studied has a more proximal 5q31 breakpoint, but she shows a similar clinical picture with some interesting differences, including the presence of microcephaly, absent in the subject studied by Abuelo *et al*[27] The *NSD1* gene, responsible, when deleted or mutated, for the Sotos syndrome with macrocephaly and excess growth, is localised in the 5q35.2 band, and it has been proposed that its duplication may cause microcephaly in the 5q35-qter duplication syndrome. The subject we studied has microcephaly, and the *NSD1* gene is active on her der(X) chromosome. Unfortunately, we have no information about *NSD1* expression in the subject described by Abuelo *et al*,[27] who did not present microcephaly.

Inactivation studies of the trisomic genes on chromosome 5

We have examined the degree of inactivation of the trisomic portion of chromosome 5 and demonstrated the silencing of autosomal genes located up to 34 Mb distal to the translocation breakpoint. These data demonstrate that the factors responsible for chromosome X inactivation are not strictly or only X-specific. Nonetheless, as shown in previous cases,[12] inactivation of the autosomic segment is discontinuous and incomplete, suggesting that autosomal chromatin does not efficiently transmit or maintain the inactivation signal.

Human/rodent somatic cell hybrids have been widely used to study the expression patterns of X-linked genes.[29] Although rare cases of spontaneous reactivation of normally inactive genes have been recorded,[30] generally the somatic cell hybrid system accurately reflects the inactivation pattern of X-linked genes on the human parental line.[29] The three genes showing discordant expression in the two der(X)-containing somatic cell hybrid clones (Table 3) may reflect system inaccuracy, incomplete inactivation, or clonal variation in gene expression. The widespread monoallelic expression reported for human autosomal genes[31] does not correlate with either inactivation status or discordant expression of the genes we analyzed.

The existence of imprinted loci on chromosome 5 has been described,[32–33] and uniparental disomy of 5q32-qter was found in children with spinal muscle atrophy[34] and childhood-onset schizophrenia.[35] Since we could not exclude *a priori* that some of the genes in our list may be subject to imprinting, we have examined their expression in one clone containing a maternal and in another containing a paternal chromosome 5 (not shown). We found complete concordance, demonstrating that none of the genes we tested was imprinted.

We have shown that some CpG islands of genes inactivated by the translocation have become methylated, as it occurs on the inactive X chromosome. Promoter CpG island methylation might be an important component in the spreading and maintenance of Xic-driven autosomal gene silencing. We also found active genes with unmethylated CpG islands, as well as active genes with a constitutionally methylated CpG island. Besides, not all inactive genes possessed promoter CpG islands, demonstrating that Xic-driven inactivation does not simply proceed by CpG island hypermethylation.

The relative position of active and inactive genes on the translocated portion of chromosome 5 suggests that inactivation spreading does not occur with a gradient of efficiency from the point closest to the breakpoint to the farthest, the telomere. In fact, the gene closest to the breakpoint (*DDX46*) and a number of nearby genes remained active, while most of the genes we tested between 143 and 159 Mb were inactive. Obviously this situation might be the result of apparently independent selection events at different times in different cells starting from the hypothetical inactivation of the entire trisomic portion of 5q. In female mice heterozygous for a null *Atrx* allele, Muers[36] found that XCI is balanced early in embryogenesis but becomes skewed over the course of development because of selection favouring cells expressing the wildtype *Atrx* allele.

LINE1 elements have been proposed to act as 'boosters' for the spread of X chromosome inactivation.[18,19] Where LINEs are dense, Xist RNA would make contact and travel through the chromosome, although where LINEs are sparse it would lose contact and be degraded. In humans, LINE1/L1 density is significantly elevated in the 'X-conserved' region (XCR),[37] where almost all genes are inactivated, while it approximately equals autosomal density in the 'X-added' region , where many genes that escape inactivation reside.[18,38] In the mouse the same relationship holds. Recent results showed that the LINE/L1 density on the opossum X chromosome is significantly lower than in the eutherian XCR.[39] Therefore LINE1 density is unlikely to be a critical factor for X chromosome inactivation in metatheria. Since the opossum has no Xist homologue, LINE1 density increase on eutherian X chromosomes could be related to Xist's role in random chromosome X inactivation. Our data also do not support a correlation between LINE1 density and gene inactivation in X;5 translocations. CTCF binds at the boundary of domains escaping X-chromosome inactivation on the X chromosome,[20] but there was no correlation between CTCF-binding sites density and gene inactivation status.

Acknowledgements

We thank Professor L Mazzanti for referring the patient. This work was supported by Telethon – Italy Grant no GGP06208 to RG

and MCB, and by PRIN 2005-MIUR, Mariani and CARIPLO foundation to OZ.

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