An acquired translocation in JAK2 Val617Phe-negative essential thrombocythemia associated with autosomal spread of X-inactivation

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The acquired mutation Val617Phe in the tyrosine kinase JAK2 was recently identified in most but not all patients with classical myeloproliferative disorders. We describe a cytogenetic and molecular study of a JAK2Val617Phe-negative case of essential thrombocythemia harboring the acquired translocation t(X;5)(q13;q33). We show that this involves the inactive X-chromosome and is associated with silencing of autosomal genes within the adjacent 5q minus syndrome common deleted region. This is the first documented example of autosomal gene silencing adjacent to an X-autosome breakpoint in human malignancy and such a mechanism may underlie the pathogenesis of related disorders with translocations involving Xq13.

Key words: essential thrombocythemia, JAK2 Val617Phe-negative, autosomal spread of X-inactivation, 5q minus syndrome.

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The classical myeloproliferative disorders comprise polycythemia vera, essential thrombocythemia and idiopathic myelofibrosis, which share clinico-pathological features including a tendency to leukemic transformation.[1] We and others have demonstrated that the gain-of-function mutation Val617Phe in the tyrosine kinase JAK2 is present in most cases of polycythemia vera, but in only half the cases of essential thrombocythemia and idiopathic myelofibrosis, with the pathogenesis of Val617Phe -negative cases still obscure.[2-5]

The study of rare chromosomal translocations has hastened the identification of genes normally targeted by sub-microscopic mutations in inherited disorders[6] and of oncogenes relevant to hematologic malignancies.[7] To investigate the pathogenesis of JAK2Val617Phe-negative myeloproliferative disorders we studied a case of essential thrombocythemia carrying the translocation t(X;5)(q13;q33),[8] particularly as several documented translocations in myeloproliferative disorders and other clonal myeloid disorders involve band Xq13.[9,10]

Design and Methods

Samples

Blood and marrow were obtained with informed consent and processed as described elsewhere.[11] The purity of granulocytes, T cells, and platelets was >95%. DNA and RNA were prepared using standard methods.[12]

Fluoresence *in situ* hybridization (FISH) and chromosome G-banding

Metaphases were derived from bone marrow and from blood after phytohemagglutinin (PHA) stimulation. Chromosomal G-banding and FISH with human DNA clones were performed as described previously.[11]

Chromosome walking

Chromosome microdissection and amplification by degenerate oligonucleotide primer (DOP)-polymerase chain reaction (PCR) has been described elsewhwere.[13] The DOP-PCR product was used as the template for chromosome walking. For each sequence-tagged site (STS), two rounds of PCR were performed using the same primer pair with appropriate controls. Primers and conditions are available on request.

Derivation and isolation of hematopoietic cell colonies

Mononuclear cells were isolated from 20 mL blood,[11] suspended at 1 to 6×10^5 cells/mL in MethocultGF H4534 medium (Stem Cell Technologies) and incubated in 5% CO_2. After 13-14 days individual colony-forming unit granulocyte monocyte (CFU-GM) colonies were lysed in Tri-reagent (Sigma) and stored at –80°C until used.

Reverse transcriptase (RT)-PCR, DNA-PCR and rapid amplification of cDNA ends (RACE)

RT reactions were performed in a total volume of 20 μL using 1 μg of total RNA or the entire RNA from a CFU-GM as described by Li *et al.*[14] The RT-PCR and DNA-PCR procedures used were those reported by Bench *et al.*[11] The HDAC8 RT-PCR primers were AGATGAAGCATCTGGTTTTT (P1) and TGGGATCTCAGAGGATAGTG (P2). 5' and 3'-RACE-ready cDNA were prepared accord-

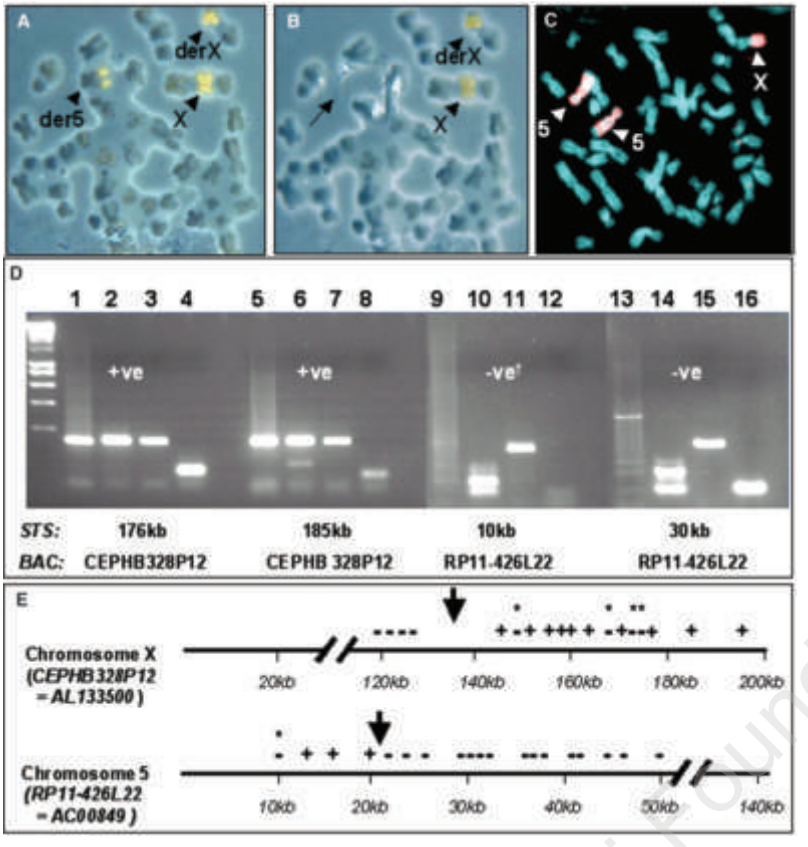

Figure 1. Molecular mapping of the t(X;5)(q13;q33) breakpoint. **A.** Phase-contrast image of FISH with whole chromosome X paint highlights the normal X, derX and der5 chromosomes in a t(X;5)(q13;q33)-carrying metaphase (arrowheads). (**B**) Same metaphase as (**A**) after microdissection of the der5 (arrow). (**C**) Reverse FISH of labeled microdissected material from (b) against a normal male metaphase shows hybridization to chromosome 5 (except its distal long arm) and the long arm of chromosome X, as expected for der5. (**D**) Examples of chromosome walking by sequence-tagged-site PCR (STS-PCR). For each STS-PCR experiment the four lanes represent i) first round PCR (DOP-PCR product as template), ii) second round PCR, iii) genomic DNA PCR (positive control) and 4) water (negative control). The location of the STS on the corresponding BAC/GenBank sequence is indicated in kilobases (kb). Lanes 1-4 and 5-8 show positive results (STS present within microdissected material). Lanes 9-12 and 13-16 show negative results (STS absent from microdissected material). Note that negative control lanes commonly showed non-specific short PCR products, presumed to represent primer multimers resulting from serial amplifications with the same primer pair. (**E**). Summary of chromosome walking by STS-PCR around the breakpoint regions of chromosome X (AL133500) and chromosome 5 (AC00849). The breakpoint locations were narrowed to regions of 15 kb on chromosome X and 2 kb on chromosome 5 (arrows), thus enabling long-range PCR across the der5 breakpoint. The presence of presumed false negative results (marked *), including the example† in lanes 9-12 in (**D**), are in keeping with the anticipated incomplete amplification of microdissected DNA by DOP-PCR.

ing to the manufacturer's instructions using the SMART RACE II kit (Clontech). Primers and conditions are available on request.

Methylation-sensitive Southern blotting

Southern blotting was performed using standard methods[12] with the probe generated by PCR (primers: TGGTCTTTCATCCCGACTTC and ATCTGCCAAACCCTTTTCCT).

Results and Discussion

A 47-year-old woman (EW) who presented in 1998 with localized breast carcinoma was found to have thrombocytosis and to carry t(X;5)(q13;q33) in nine of ten bone marrow metaphases. The salient features of this case have been published[8] and meet international diagnostic criteria for essential thrombocythemia.[15] The *JAK2* Val617Phe mutation was not detected in marrow nucleated cells or blood granulocytes by allele-specific PCR and *Bsa*XI digestion.[2] In 2002, the patient had a localized renal tumor removed surgically. Chromosomal fragility studies of blood lymphocytes were normal. In 2005, the patient remains free of breast and renal cancer and is taking hydroxyurea for her thrombocytosis.

Using G-banding and FISH, t(X;5)(q13;q33) was identified in 58 of 60 bone marrow metaphases derived 4 years after diagnosis, but not in 150 blood PHA-derived and 250 skin fibroblast metaphases, confirming that the translocation was acquired (*data not shown*). A trephine biopsy revealed hypolobated megakaryocytes, a feature of 5q minus syndrome (*see online appendix*). FISH with human DNA clones localised the chromosome 5 breakpoint to BAC RP11-426L22 (AC008491) and the chromosome X breakpoint to BAC CEPHB328P12 (AL133500) (*see online appendix*). To define the breakpoints molecularly, a single derivative chromosome 5 (der5) was microdissected (Figures 1A and 1B) and amplified by DOP-PCR. Correct microdissection was confirmed by back-hybridizing labeled DOP-PCR product onto a normal metaphases (Figure 1C). The breakpoint region was narrowed using chromosome walking (Figures 1D and 1E) and the breakpoint cloned from granulocyte DNA by long-range PCR: chromosome 5 sequences finished at nucleotide 21515 of AC008491 and chromosome X sequences started at nucleotide 138072 of AL133500. PCR across the derX breakpoint gave a product of the expected size, confirming a reciprocal translocation without large deletions adjacent to the breakpoint (*data not shown*).

The map of der5 in Figure 2A highlights two significant consequences of the t(X;5)(q13;q33): (i) the *HDAC8* gene is physically disrupted and (ii) the common deleted region (CDR) for 5q minus syndrome lies within 6 Mb of the X-inactivation center (X_{IC}). 5' and 3' RACE on marrow and blood cDNA led to isolation of full length

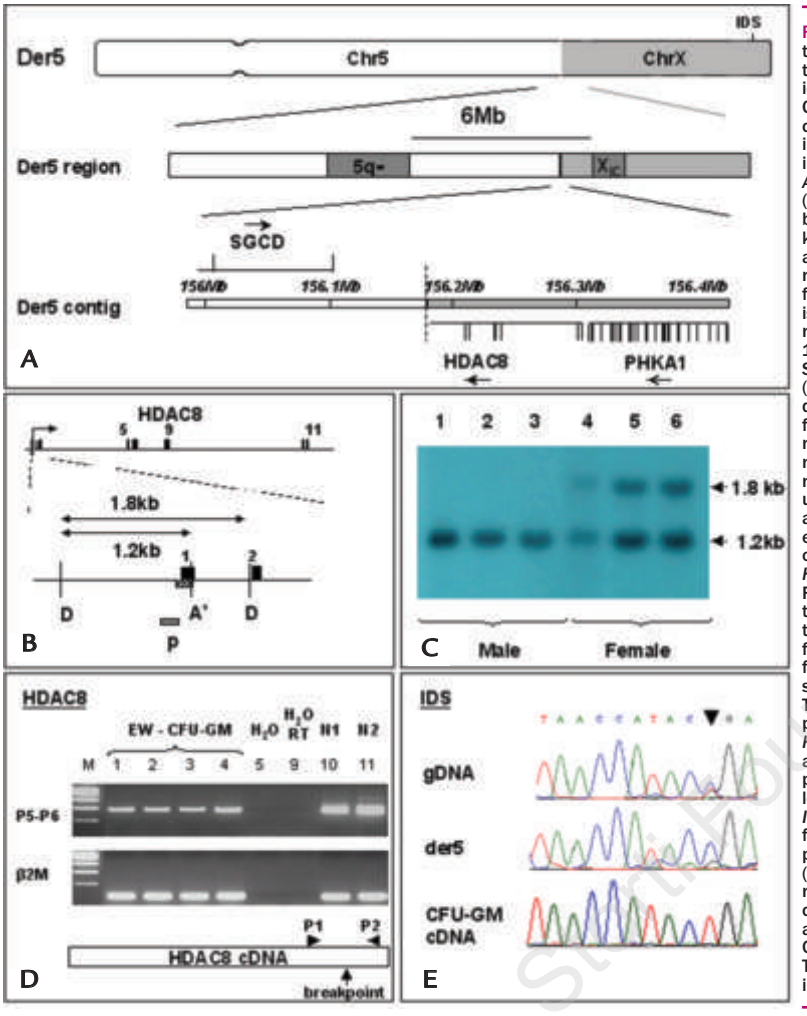

Figure 2. The inactive X-chromosome is involved in t(X;5)(q13;q33). (A) Map of the breakpoint region on the derivative chromosome 5 (Der5): *HDAC8* is physically disrupted on chromosome X and the 5q minus CDR (5q-) comes to lie 6 Mb from the X-inactivation centre (X_{IC}). The chromosome 5 breakpoint is located in intergenic DNA and does not directly disrupt the intron-exon structure of any known or predicted gene. *Arrows indicate the direction of gene transcription*. (B) Design of methylation-sensitive genomic Southern blot for *HDAC8*: DraI (D) digestion generates an 1.8 kb restriction fragment containing the *HDAC8* exon 1 and the associated CpG island (shaded box). The methylation-sensitive AvaI (A*), cleaves this into two fragments (1.2 kb and 0.6 kb) when the *HDAC8* CpG island is unmethylated but leaves the 1.8 kb fragment intact when the CpG island is methylated. The 1.8 kb and 1.2 kb fragments can be detected by Southern hybridization with the same DNA probe (p). (C) Southern hybridization shows complete *AvaI* digestion at the *HDAC8* CpG island of leukocyte DNA from normal males (lanes 1-3) (only the 1.2 kb fragment seen) but incomplete digestion of that from normal females (lanes 4-6) (1.8 kb and 1.2 kb fragments), even when a smaller quantity of DNA was used (lane 4). Thus, females carry both methylated and unmethylated copies of the CpG island associated with the gene's promoter, whereas males carry only an unmethylated allele, demonstrating that *HDAC8* is subject to X-inactivation in females. (D) Four examples of individual CFU-GM colonies carrying t(X;5)(q13;q33) (verified by PCR amplification across the der5 breakpoint, *data not shown*), were studied for *HDAC8* mRNA expression by RT-PCR using primers from exons lying on opposite sites of the X-chromosome breakpoint (P1 on exon 9 and P2 on exon 11). The gene for β2-microglobulin (β2M) was used as a positive control. All four colonies expressed intact *HDAC8* mRNA. (E) *IDS* is located on the distal long arm of the normal X-chromosome and is therefore present on der5 (*see Figure 2A*). PCR and RT-PCR, followed by direct sequencing were used to identify the *IDS* allele present. Both C and T alleles were amplified from genomic DNA (gDNA), confirming that our patient is heterozygous for this polymorphism (dbSNP: 1141608). The allele amplified from the microdissected der 5 and therefore present on the X-chromosome involved in t(X;5)(q13;q33) is the C allele. All 12 individual t(X;5)(q13;q33)-carrying CFU-GM (example shown), expressed the mRNA from the T allele, confirming that the inactive X chromosome is involved in the translocation.

HDAC8 and a splice variant (GenBank, BE792074), but no fusion or abnormal *HDAC8* transcripts (*data not shown*). *PHKA1* and *RPS4X*, two genes that flank *HDAC8*, are discordant with regards to X-inactivation in humans,[16] but the status of *HDAC8* has not previously been determined. We show, using methylation-sensitive genomic Southern blotting, that *HDAC8* is subject to X-inactivation (Figure 2B and 2C).

To examine whether the active (X_a) or inactive (X_i) X chromosome is involved in t(X;5)(q13;q33), we studied *HDAC8* mRNA expression in individual translocation-carrying (verified by PCR) CFU-GM colonies. Using *HDAC8* RT-PCR primers on opposite sites of the chromosome 5 breakpoint, *HDAC8* mRNA was amplified from all colonies tested (Figure 2D), demonstrating that its intact (*non-translocated*) allele is transcriptionally active. This suggests that either Xi is involved in the translocation or that X_a is involved with subsequent reactivation of $HDAC8_i$ on X_i. To distinguish these possibilities, we studied an expressed polymorphism (C/T) in the iduronate-2-sulphatase gene (*IDS*) (dbSNP: 1141608), located on the distal long arm of chromosome X and thus on der5. We show that der5 carries the C allele, whilst only the T allele is expressed in 12 of 12 t(X;5)(q13;q33)-carrying CFU-GM (Figure 2D). This demonstrates that der5 carries a transcriptionally silent IDS_i allele and therefore that X_i is involved in the translocation.

Since the t(X;5)(q13;q33) places the 5q minus CDR within 6 Mb of the X_{IC} and given the presence of histological features of this syndrome in the marrow of our patient, we speculated that X-inactivation spread to autosomal genes in this CDR. We therefore studied expression of informative/polymorphic genes in the region of the CDR in individual, translocation-carrying, CFU-GM colonies. The woman was heterozygous for expressed polymorphisms in four genes: *TCOF1*, *HSPA9B*, *G3BP* and *GM2A* (Figure 3A). *TCOF1* and *HSPA9B* were expressed biallelically in CFU-GM (*data not shown*). By contrast, *G3BP* and *GM2A* showed monoallelic silencing ((Figure 3B). In the case of *G3BP*, we show that the silent allele was carried on der5. These data are consistent with silencing of chromosome 5 genes by patchy spread of X-inactivation as described in reports of inherited X-autosome translocations.[17]

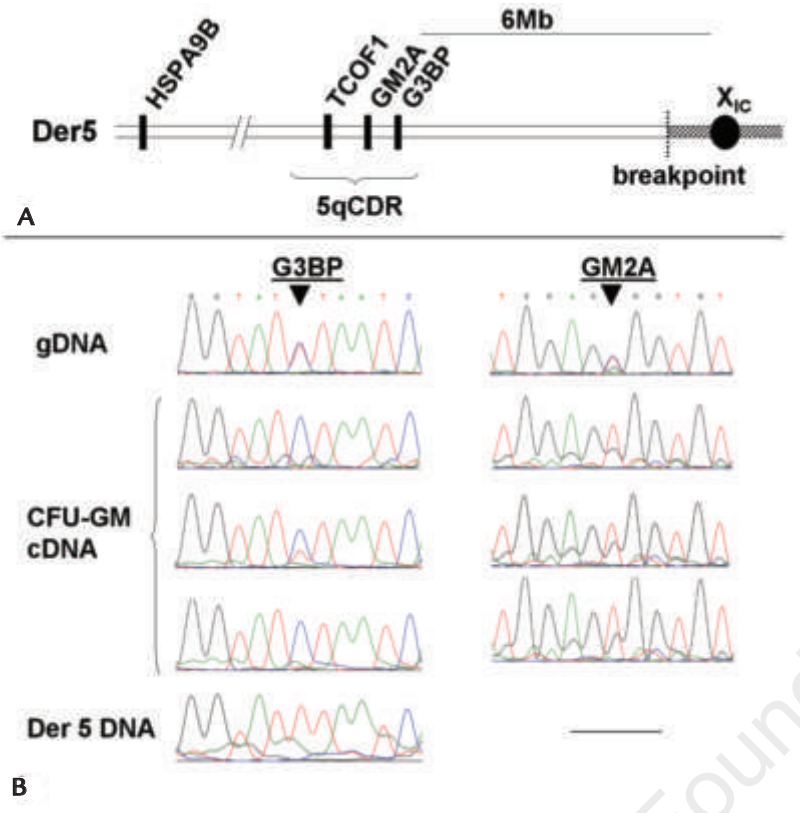

Figure 3. Evidence for autosomal spread of X-inactivation. (A) Map of the der5 breakpoint region showing the distance of the genes *G3BP*, *GM2A*, *TCOF1* and *HSPA9B* from the X-inactivation center (X_{IC}). The patient was found to be heterozygous for expressed polymorphisms in these genes after screening for several known polymorphisms in the region. (B) Both alleles of *GM2A* and *G3BP* (C/T) were amplified from the patient's genomic DNA (gDNA) while her CFU-GM colonies expressed only one of the two alleles by RT-PCR (*G3BP-C* and *GM2A-T*). For *G3BP* we were able to amplify the allele carried by der5 (Der 5 DNA) and type it as the T allele. This was not possible for *GM2A*, presumably as a result of incomplete amplification of der5 by DOP-PCR as was seen for some STSs used for chromosome walking. (Figure 1E) *TCOF1* and *HSPA9B* were expressed biallelically (*data not shown*).

We report the first case of a somatic clonal translocation involving Xi associated with persistent silencing of autosomal genes. Spread of X-inactivation up to 45 Mb away from an X-autosome junction has been described in inherited X-autosome translocations.[17] Such translocations are established prior to initiation of X-inactivation and autosomal genes are thought to be silenced by an otherwise physiological process extending beyond the X-autosome junction. By contrast in somatic cells X-inactivation is thought to be transcriptionally stable and persists even when the X_{IC} is removed[18] and although autosomal X-inactivation has been induced experimentally in somatic cell lines by inserting XIST transgenes into autosome,[19] it was progressively lost in culture. In the case described here, epigenetic silencing of genes was present several years after the translocation arose suggesting it was advantageous.

Juxtaposition of the X_{IC} next to the 5q minus CDR and the presence of histological features of this syndrome raised the possibility that silencing of autosomal genes through spread of X-inactivation contributed to our patient's clonal disorder. In keeping with this, we have shown that genes in the CDR are silenced. It is noteworthy that the CDR genes studied were picked solely because they were informative in our patient. This makes a *field* effect (i.e. spread of X-inactivation) more likely to be operative than disruption of gene-specific regulatory elements by the translocation as has been described for translocations between autosomes.[20]

Spread of X-inactivation may not be unique to this case as there are several reports of patients with clonal myeloid disorders carrying chromosomal translocations between Xq13 and autosomal regions such as 5q33 and 20q13 that are recurrently deleted in clonal myeloid disorders.[9,10,21,22] Furthermore, a search of the Mitelman Database (*http://cgap.nci.nih.gov/Chromosomes/Mitelman*) for clonal bone marrow disorders carrying t(X;5) or t(X;20) identified 24 cases, all in females, suggesting a requirement for the presence of X_i. Our data support a novel mechanism of leukemogenesis, namely the epigenetic silencing of autosomal genes as a result of spread of X-inactivation in Xi - autosome translocations.

GSV and PJC contributed to the design, experimental conduct, analysis and writing of this work. JL, IR, SS, BJPH, NF and EJB contributed to experimental conduct. LRM and DAC conducted case ascertainment and sample collection. LMS and ARG contributed to study design, analysis and writing. The authors thank Dr. Ralf Sudbrak for CEPHB BACs, Professor Nicholas Cross for cosmids Km4.1 and Km9.4, the Wellcome Trust Sanger Institute for all other BACs and David Stevenson, Aberdeen RI, for skin metaphase analysis. Funding: GSV and PJC were funded by Leukaemia Research Fund Clinical Training Fellowships. Work in the authors' laboratories is funded by the Leukemia Research Fund (UK) and the Wellcome Trust.

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