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

The classical myeloproliferative disorders comprise polycythemia vera, essential thrombocythemia and idiopathic myelofibrosis, which share clinicopathological features including a tendency to leukemic transformation. 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. The study of rare chromosomal translocations has hastened the identification of genes normally targeted by sub-microscopic mutations in inherited disorders and of oncogenes relevant to hematologic malignancies. To investigate the pathogenesis of JAK2 Val617Phe-negative myeloproliferative disorders we studied a case of essential thrombocythemia carrying the t(X;5)(q13;q33), particularly as several documented translocations in myeloproliferative disorders and other clonal myeloid disorders involve band Xq13.

Design and Methods

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

Fluorescence 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.

Chromosome walking
Chromosome microdissection and amplification by degenerate oligonucleotide primer (DOP)-polymerase chain reaction (PCR) has been described elsewhere. 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, suspended at 1 to 6×10⁶ cells/mL in MethocultGF H4534 medium (Stem Cell Technologies) and incubated in 5% CO₂. 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. The HDAC8 RT-PCR primers were AGATGAAGCATCTGGTTTTT (P1) and TGGGATCTCAGAGGATAGTG (P2). 5’ and 3‘-RACE-ready cDNA were prepared accord-
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 with the probe generated by PCR (primers: TGGTCTTTCATCCCGACTTC and ATCTGCCAAACCTTTTCCT).

**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 and meet international diagnostic criteria for essential thrombocythemia. The JAK2 Val617Phe mutation was not detected in marrow nucleated cells or blood granulocytes by allele-specific PCR and BsaXI digestion. 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 hypopoplated 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 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.

**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 iv) 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.
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, 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 (Xa) or inactive (Xi) 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 Xa is involved with subsequent reactivation of HDAC8 on X. 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 allele and therefore that Xa is involved in the translocation.

Since the t(X;5)(q13;q33) places the 5q minus CDR within 6 Mb of the Xc 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: TCOFI, HSPAp9B, G3BP and GM12A (Figure 3A). TCOFI and HSPAp9B were expressed biallelically in CFU-GM (data not shown). By contrast, G3BP and GM12A 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.
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. 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 XIC is removed and although autosomal X-inactivation has been induced experimentally in somatic cell lines by inserting XIST transgenes into autosome, 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 XIC 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.

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.

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. 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.

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