

## LETTER TO THE EDITOR

Inactivation of *BANK1* in a novel *IGH*-associated translocation t(4;14)(q24;q32) suggests a tumor suppressor role in B-cell lymphoma

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Immunoglobulin heavy chain (*IGH*) gene-associated translocations occur frequently in different subtypes of B-cell lymphomas.<sup>1</sup> These translocations result in deregulated expression of partner genes and play pivotal roles in the pathogenesis of lymphomas. Molecular characterization of *IGH*-associated translocation breakpoints has been instrumental in the identification of genes important to the development of normal and malignant B cells.

We identified a novel balanced reciprocal translocation, t(4;14)(q24;q32), by conventional karyotyping (Figure 1a) in a case of post-transplant lymphoproliferative disorder (PTLD). The patient underwent stem cell transplantation for classical Hodgkin's lymphoma and developed a gastric mass that was diagnosed as Epstein-Barr virus (EBV)-positive polymorphic PTLD with monomorphic areas. Fluorescence *in situ* hybridization (FISH) with *IGH* dual-color probe demonstrated that the translocation involved the *IGH* locus (Figure 1b). The breakpoint sequence was cloned by long-distance inverse PCR<sup>2-4</sup> and sequence analysis identified the partner gene as *BANK1* (*B-cell scaffold protein with ankyrin repeats 1*) (Figure 1c). The breakpoints were located in the switch region ( $S\alpha$ ) proximal to the  $C\alpha 2$  gene segment near the 3' end of *IGH*, and in intron 1 of the *BANK1* gene ~830 bp telomeric to the 3' end of exon 1a. The juxtaposed *IGH* and *BANK1* have opposite transcription orientations with a head-to-head configuration.

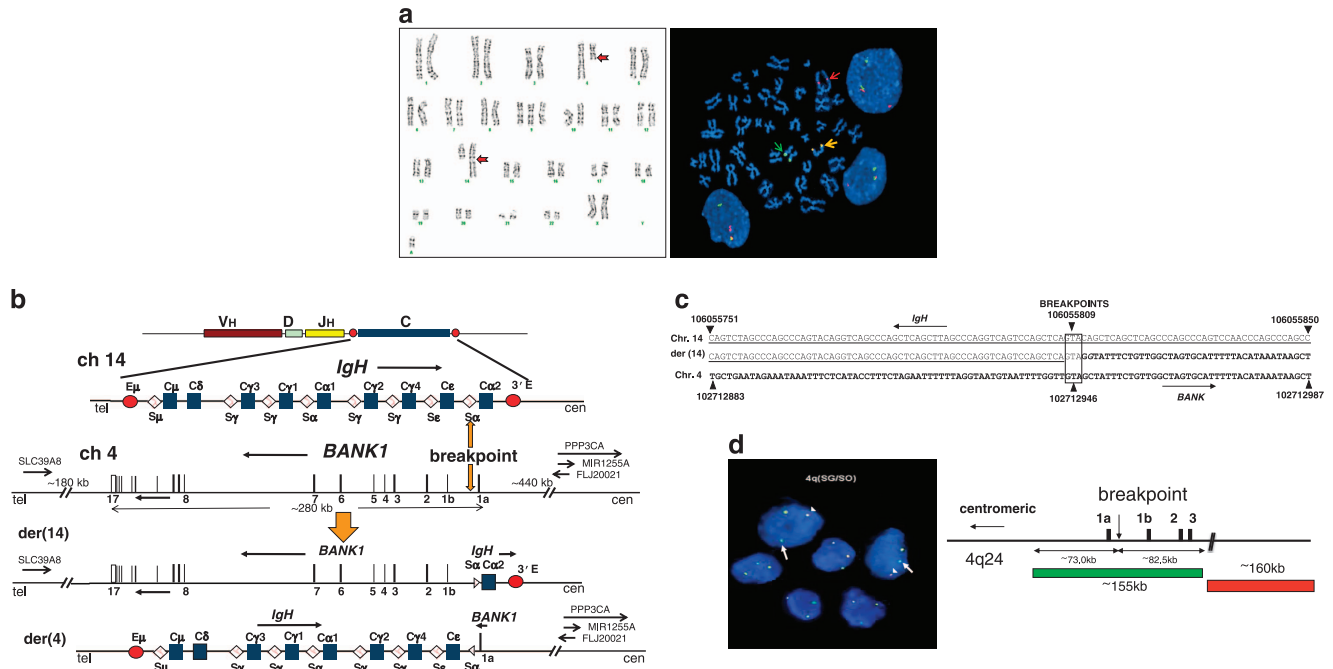
*IGH/BANK1* translocation was further confirmed by FISH using double-color break-apart assay with two BAC probes, RP11-96J17 and RP11-138I19, performed on cells isolated from the PTLD case (Figure 1d). We screened an additional 68 diffuse large B-cell lymphomas (DLBCLs) and 15 PTLDs by FISH using the same probes. None of them were found to harbor any translocations affecting the *BANK1* locus (data not shown), suggesting that translocation involving *BANK1* is a rare event.

*BANK1* acts as an important adaptor molecular to link B-cell receptor (BCR)-mediated signaling to the generation of intracellular secondary messengers in B cells. Phosphorylated *BANK1* enhanced BCR-induced calcium mobilization by binding to LYN, thus promoting LYN-mediated tyrosine phosphorylation of 1,4,5-triphosphate receptor (IP3R) that leads to release of calcium from intracellular stores.<sup>5</sup> *BANK1* has recently been shown to physically interact with B-cell lymphoid kinase (BLK),<sup>6</sup> another B-cell specific Src family kinase, and phospholipase C  $\gamma 2$  (PLC $\gamma 2$ ),<sup>7</sup> a major molecular switch in B-cell signal transduction, upon BCR engagement. In addition to an enhancing function in BCR signaling, *BANK1* may have an inhibitory role in the CD40 signaling pathway. Studies on *BANK1*-deficient mice suggested that *BANK1* attenuates CD40-mediated AKT activation to prevent hyperactive B-cell responses.<sup>8</sup> *BANK1* single-nucleotide polymorphic variants have also been implicated in autoimmune diseases such as systemic lupus erythematosus.<sup>9</sup> Although evidence of an important function of *BANK1* in B-cell physiology is emerging, a role for *BANK1* in lymphoma development has not been reported previously.

*BANK1* encodes three RNA isoforms, 1a, 1b and  $\Delta 2$ , with 1a being the major isoform.<sup>5,9</sup> The two full-length isoforms, 1a and 1b, are derived from alternative promoter usage that results in the generation of exon 1a or 1b. The  $\Delta 2$  isoform contains an in-frame deletion of exon 2. *BANK1* is mainly expressed in immature and mature B cells with very low expression in pro B cells and absent expression in T cells. To determine whether *BANK1* expression is differentially expressed during B-cell differentiation, B-cell subsets including naive, germinal center (GC) and memory B cells were isolated and *BANK1* mRNA expression was determined by real-time quantitative reverse transcriptase-PCR (qRT-PCR). All *BANK1* isoforms were expressed at higher levels (2.5–8 fold) in the naive and memory B cells compared with the GC B cells (Figure 2A). In line with this, immunohistochemistry (IHC) on reactive tonsils showed a distinctly lower level of expression in the GCs and higher levels in the mantle zones (Figure 2A). These findings imply a physiological downregulation of *BANK1* expression during GC transit.

To determine the effect of the t(4;14) translocation on *BANK1* expression, real-time qRT-PCR was performed to quantitate total *BANK1*, total full-length *BANK1* (that is, 1a plus 1b) and the three individual *BANK1* isoforms (1a, 1b and  $\Delta 2$ ) mRNA levels. Interestingly, we did not detect evidence of activation of *BANK1* expression by the *IGH* translocation. Instead, we observed downregulation of *BANK1* expression as a result of the translocation. The expression for total and full-length *BANK1* mRNAs was greatly reduced (>90%) in the PTLD case compared with GC B cells (Figure 2B). *BANK1* 1a and  $\Delta 2$  transcript isoforms, both initiated from the exon 1a promoter, were only barely detectable. The level of *BANK1* 1b transcript isoform was ~40% of the normal GC B cells. These results are consistent with the loss of expression of the major *BANK1* 1a isoform because of dissociation of exon 1 from the rest of the *BANK1* gene, and concurrent silencing of the nontranslocated *BANK1* allele. The latter allele is grossly intact, as implied by FISH, and we have excluded the possibility of hypermethylation (data now shown), suggesting other unknown mechanisms are responsible for repressing *BANK1* transcription from the nontranslocated allele. It is noteworthy that although the translocated *IGH* locus is in a favorable position to do so, we did not observe activation of transcription initiated from the adjacent alternative *BANK1* exon 1b promoter or from an intronic cryptic promoter. IHC confirmed the lack of *BANK1* in the tumor cells (Figure 2B). In contrast, we detected variable *BANK1* expression by IHC in other (8 of 8) PTLDs, 4 of 4 EBV-positive DLBCLs of the elderly and 48 of 54 DLBCL, not otherwise specified (DLBCL-NOS), all of which did not harbor *BANK1* rearrangements (Supplementary Table 1 and Figure 2C). Thus, we believe that the main consequence of *IGH* translocation to the *BANK1* locus is most likely downregulation of *BANK1* through dissociation of the major promoter. Of the 54 DLBCLs, 6 also lacked *BANK1* expression, suggesting the presence of alternative mechanism(s) of *BANK1* downregulation in B-cell lymphomas.

Target gene downregulation is a highly unusual consequence of *IGH* translocations, as they have been shown to lead to overexpression of the target gene in almost all circumstances.



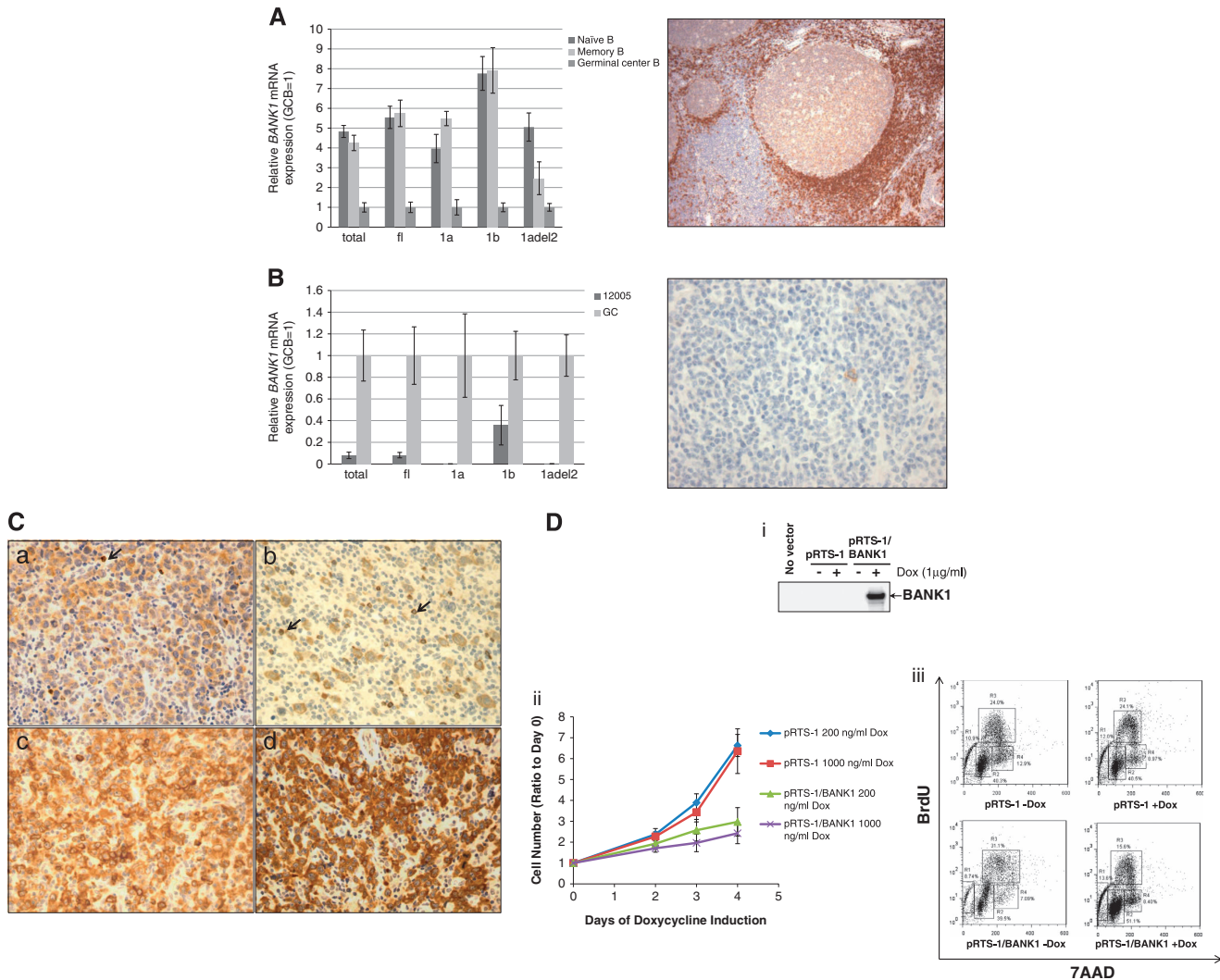
**Figure 1.** Identification of a novel translocation  $t(4;14)(q24;q32)$  and identification of *BANK1* as an *IGH*-associated partner. **(a)**, (left) Karyotype showing the  $t(4;14)(q23;q32)$  translocation and trisomy 9. **(a)**, (right) Metaphase and interphase FISH assay showing rearrangement of the *IGH* gene. Fusion (yellow) signal indicates the normal *IGH* allele and the two arrows show the split signals of *IGH* on the der(4) and der(14) indicating the rearrangement of the gene. **(b)** The structures of the germline *IGH* and *BANK1* alleles, as well as the translocated *IGH/BANK1* allele in der(14) and der(4), are depicted. Vertical boxes denote exons of the *BANK1* gene. Sequence analysis of the breakpoint region showed that the *BANK1* gene is juxtaposed to *IGH*. The breakpoint is located at the switch (S) region proximal to the  $\text{Ca}2$  gene segment at the 3' portion of the *IGH* gene, and in intron 1 of the *BANK1* gene, ~830 bp telomeric to the 3' end of exon 1a. *IGH* and *BANK1* are arranged in a head-to-head transcription orientation. This *IGH/BANK1* translocation results in removal of the major *BANK1* promoter from the rest of the gene. Four neighboring coding genes (*PPP3CA*, *MIR1255A*, *FLJ20021* and *SLC39A8*) are shown by horizontal arrows, the direction and length of which represent the orientation and size of the transcription units. The approximate genomic distance between the 5' or 3' end of *BANK1* and the closest exon of the neighboring genes are indicated. C, constant; cen, centromeric; E, enhancer; S, switch; tel, telomeric. **(c)** The sequence alignment of the translocation breakpoint is shown. *IGH* sequence is underlined and *BANK1* sequence is in bold. The breakpoint is boxed (the exact nucleotide location cannot be determined). The translocated *BANK1* sequence shows a C to G mutation (in italics) in very close vicinity to the breakpoint. **(d)** Interphase nuclei were hybridized with BAC RP11-96J17 (green, centromeric) and BAC RP11-138I19 (red, telomeric) in 4q24 to confirm *BANK1* rearrangement. The arrow indicates the green signal present on derivative chromosome 4 generated by a split within RP11-96J17. The arrowhead points to the signal generated from the remainder of BAC RP11-96J17 and the adjacent RP11-138I19, present on derivative chromosome 14. The yellow signal (not marked) in the cells corresponds to the intact, untranslocated *BANK1* allele. Only exons 1a, 1b, 2 and 3 of *BANK1* are shown.

*IGH*-mediated gene inactivation has been implicated only once before in hematopoietic malignancies for the  $t(14;16)(q32;q23)$  translocation that is found in <5% of plasma cell myelomas.<sup>10</sup> All the breakpoints of this translocation are located in the last intron of the tumor suppressor gene *WWOX* (*WW domain containing oxidoreductase*).<sup>11,12</sup> This intron is nearly 1-Mb long and contains the fragile site FRA16D.<sup>13</sup> *IGH* in this translocation is thought to have a dual pathogenetic function. It is in a position to disrupt the *WWOX* gene and prevent the generation of a full-length protein and, at the same time, causes overexpression of the neighboring *MAF* proto-oncogene located telomeric to *WWOX* via the *IGH* enhancer.<sup>14</sup>

We have also considered the remote possibility that the *IGH* translocation activates a gene in the vicinity of *BANK1*. Three coding genes, *PPP3CA*, *MIR1255A* and *FLJ20021*, reside ~443 kb centromeric to the *BANK1* breakpoint, and one coding gene, *SLC39A8*, is located ~460 kb telomeric to the *BANK1* breakpoint (<http://www.ncbi.nlm.nih.gov/gene/55024>) (Figure 1b). *PPP3CA*, *MIR1255A* and *SLC39A8* have the same orientation as the translocated *IGH*, whereas *FLJ20021* has the opposite orientation to the translocated *IGH*. It is unlikely that the translocated *IGH* on der(4) drives expression of any of these three genes using the germline I transcript promoter because of their considerable

distance from the IgH and/or incompatible relative transcriptional orientation. Furthermore, the configurations of the translocated *IGH* relative to these genes, either upstream/same orientations for *PPP3CA* and *MIR1255A* or downstream/opposite orientations for *FLJ20021*, are not considered favorable for an enhancer activation mechanism. Although *IgH* is located downstream of *SLC39A8* in the same orientation and can conceivably activate the latter through its 3' enhancer on the der(14) chromosome, *SLC39A8* mRNA level in this case was not significantly higher compared with EBV-positive B-cell lymphomas without the *IGH/BANK1* translocation (data not shown). Thus, there is no definitive evidence for activation of *SLC39A8* by the translocated *IGH* locus.

The inactivation of *BANK1* in a *IGH*-associated translocation suggests that abnormal downregulation of *BANK1* can promote lymphoma development. To show directly that *BANK1* has a negative inhibitory effect on lymphomagenesis, we transfected BC3 primary effusion lymphoma cells with an all-in-one doxycycline-inducible expression vector pRTS-1,<sup>15</sup> carrying *BANK1* complementary DNA to establish an inducible *BANK1*-expressing cell line. The BC3 cell line was chosen because it has no detectable endogenous *BANK1*. Addition of doxycycline resulted in efficient induction of *BANK1* and a significant retardation in the rate of cell number increase compared with the control transfectants



**Figure 2.** *IgH* translocation of *BANK1* resulted in its downregulation and may contribute to lymphoma development by promoting cell proliferation. **(A, left)** Levels of different *BANK1* mRNA isoforms: total, full-length (fl), isoform 1a (1a), isoform 1b (1b) and  $\Delta 2$  isoform (1adel2) were measured by qRT-PCR and expressed relative to the normal GC B cells. *BANK1* mRNA expression in naive and memory B cells is significantly higher compared with that in GC B cells. **(A, right)** IHC was performed on paraffin sections of a reactive tonsil using a polyclonal antibody against *BANK1*. *BANK1* is weakly expressed in GC but strongly expressed in the mantle zones (MZ). T cells are negative for *BANK1*. **(B, left)** *BANK1* mRNA isoforms were determined in the lymphoma case (12005) harboring *BANK1* translocation by qRT-PCR as described in **(A)**. *BANK1* expression is decreased compared with GC B-cells. In particular, isoforms 1a as well as  $\Delta 2$  are virtually absent, mostly likely because of dissociation of exon 1-initiated promoter from the rest of the *BANK1* gene in the translocated allele and silencing of the nontranslocated allele. **(B, right)** IHC was performed on paraffin sections of the lymphoma case (12005) using a polyclonal antibody against *BANK1*. The tumor cells are negative for *BANK1*. A rare *BANK1*-positive tumor cell is shown as an internal control. **(C)** Variable expressions of *BANK1* in PTLD and DLBCL. (a) Weak expression in a monomorphic PTLD; (b) weak expression in a polymorphic PTLD; (c) moderate expression in a monomorphic PTLD; (d) strong expression in a DLBCL-NOS. Arrows indicate normal reactive B cells that show distinctly higher *BANK1* expression compared with the weak *BANK1*-expressing lymphoma cells. **(D)** *BANK1* has a negative effect on cell cycle. **(Di)** BC3 primary effusion lymphoma (PEL) cells stably transfected with pRTS-1/*BANK1* expressed *BANK1* in the presence of doxycycline (Dox). **(Dii)** Overexpression of *BANK1* in BC3 cells upon Dox addition resulted in a slower growth rate compared with controls. **(Diii)** Bromodeoxyuridine (BrdU) incorporation assay showed that *BANK1* caused a decrease in cell proliferation by 50%.

(Figure 2Di and ii). Bromodeoxyuridine staining demonstrated a decreased incorporation from 31.1 to 15.6% upon *BANK1* expression, implying a negative effect of *BANK1* on cell cycle (Figure 2Diii).

This study identified *BANK1* as a novel *IGH* translocation partner and demonstrated a rare mechanism of gene inactivation by *IGH* through promoter dissociation. Our initial functional studies demonstrating a negative effect of *BANK1* on cell proliferation suggest that *BANK1* inactivation may contribute to lymphoma by facilitating cell proliferation. Unlike the myeloma-associated t(14;16)(q32;q23), which has a dual impact on two neighboring genes (*WWOX* and *MAF*), the impact of (4;14) translocation

described here appears to be restricted to *BANK1*. Our study underscores the utility of cloning *IGH*-associated translocations, even the rare ones, to identify genes with important functions in normal and neoplastic B-cell biology. We provide, for the first time, genetic and functional data suggesting a possible tumor suppressor role of *BANK1* in B-cell lymphomagenesis. Further studies are warranted to further investigate the involvement of *BANK1* in mature B-cell malignancies.

#### CONFLICT OF INTEREST

The authors declare no conflict of interest.



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