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The present invention relates to an episomal structure expressing a functional oncogene, whereby said oncogene is a fusion gene of two chromosomal gene fragments. More specifically, the invention relates to a NUP214-ABL1 fusion product, important in the development of T-cell acute lymphoblastic leukemia, to methods to detect the fusion and to methods to prevent the oncogenic activity of said fusion product.

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(54) Title: EPISOMAL FUSION GENE

(57) Abstract: The present invention relates to an episomal structure expressing a functional oncogene, whereby said oncogene is a fusion gene of two chromosomal gene fragments. More specifically, the invention relates to a NUP214-ABL1 fusion product, important in the development of T-cell acute lymphoblastic leukemia, to methods to detect the fusion and to methods to prevent the oncogenic activity of said fusion product.

EPISOMAL FUSION GENE

The present invention relates to a mammalian episomal structure expressing a functional oncogene, whereby said oncogene is a fusion gene of two chromosomal genes. More specifically, the invention relates to a NUP214-ABL1 fusion product, important in the development of T-cell acute lymphoblastic leukemia, to methods to detect the fusion and to methods to prevent the oncogenic activity of said fusion product.

Acute leukemia is a disease of the leukocytes and their precursors. It is characterized by the appearance of immature, abnormal cells in the bone marrow and peripheral blood. The acute leukemia are classified according to morphological, cytochemical and immunological criteria.

T-cell acute lymphoblastic leukemia (T-ALL) accounts for about 10 to 15% of newly diagnosed cases of childhood acute lymphoblastic leukemia, and children with T-ALL generally have a poorer prognosis than those with precursor B-lineage ALL (B-ALL). As several genetic defects have been identified as cause of T-ALL, individualization of T-All treatment might improve outcome and long-term quality of life. Despite a detailed understanding of deregulated transcription factors in T-cell acute lymphoblastic leukemia (T-ALL), mutations in protein tyrosine kinases have only rarely been identified in this disease (Fernando *et al.*, 2002; Pui *et al.*, 2004; Paietta *et al.*, 2004).

In this invention we describe the episomal (Maurer *et al.*, 1987) amplification of *ABL1* in 5 of 90 (5.6 %) T-ALL patients, an aberration that is not detectable by conventional cytogenetics normally used for typing T-ALL mutations, but can be detected by all techniques that can identify the episomal structure, such as FISH, micro-array-based comparative genomic hybridisation or PCR. Molecular analyses delineated the amplicon as a 500 kb region from 9q34, containing the oncogenes *ABL1* and *NUP214* (de Klein *et al.*, 1982; von Lindren *et al.*, 1992). Surprisingly, a previously undescribed mechanism for activation of tyrosine kinases in cancer was identified: formation of episomes resulting in the generation of a fusion between *NUP214* and *ABL1*. The *NUP214-ABL1* transcript was detected in 5 patients with the *ABL1* amplification, in 5 of 85 (5.8 %) additional T-ALL patients, and in 3 of 22 T-ALL cell lines. The constitutively phosphorylated tyrosine kinase NUP214-ABL1 is sensitive to the tyrosine kinase inhibitor imatinib (Capdeville *et al.*, 2002). The recurrent cryptic *NUP214-ABL1* rearrangement is associated with increased *HOX* expression (Fernando *et al.*, 2002) and deletion of *CDKN2A* (Hebert *et al.*, 1994), consistent with a multi-step pathogenesis of T-ALL. *NUP214-ABL1* expression defines a new subgroup of T-ALL patients that could benefit from imatinib treatment.

A first aspect of the invention is an isolated NUP214-ABL1 fusion gene. Preferably, said fusion gene expresses a tyrosine kinase, even more preferably, said tyrosine kinase is constitutively expressed. Most preferably, said fusion gene plays a role in the development of leukemia. Preferably, said leukemia is selected from the group consisting of T-ALL, B-ALL precursor B-

LL and *BCR-ABL1* negative myeloproliferative diseases. Most preferably, said leukemia is T-ALL.

In a preferred embodiment, said fusion gene is situated on a mammalian episomal structure. Said mammalian episomal structure is consisting of a chromosomal fragment, comprising the fusion of at least NUP214 and ABL1, whereby said fusion is preferably actively expressing a fusion oncogene. A chromosomal fragment, as used here, means that the DNA is normally present on the non-mutated mammalian chromosome. Said chromosomal fragment preferably does not comprise DNA that is directly derived from viral DNA, by integration of viral DNA into the chromosome. Mammalian episomal structure as used here means a submicroscopic circular DNA fragment, self-replicating in mammalian cells, as described by Maurer *et al.* (1987). Preferably, said episomal structure is smaller than the microscopically visible double-minute chromosomes. Preferably, said episomal structure is smaller than 1Mb, even more preferably smaller than 750 kb, most preferably smaller than 500 kb. Mammalian episomal structures are clearly different from viral episomal structures such as HPV derived episomes.

It is known that genes can be amplified on episomal structures and double minute chromosomes. However, all genes described that are amplified this way, including known oncogenes, are genes that as such are present on the mammalian chromosome. Surprisingly we found that the formation of an episomal structure can create a novel, expressed fusion gene encoding a functional protein.

Still another aspect of the invention is a method to detect a fusion gene according to the invention. Preferably, said method comprises a technique selected from the group consisting of FISH, micro-array-CGH and PCR. It is important to note that a fusion gene, situated on a mammalian episomal structure, is normally overlooked by classical karyotyping ans special attention should be paid to detect those structures

Another aspect of the invention is a method of typing leukemia, comprising the detection of a NUP214-ABL1 fusion gene according to the invention. Preferably, said leukemia is selected from the group consisting of T-ALL, B-ALL, precursor B-LL and *BCR-ABL1* negative myeloproliferative diseases, even more preferably, said leukemia is T-ALL. Preferably, said method is allowing the detection a the fusion gene situated on a mammalian episomal structure

Another aspect of the invention is a method to inhibit a constitutively activated kinase expressed by a NUP214-ABL1 fusion gene. Preferably, said method comprises the use of imatinib (also known as imatinibe mesylate, Glivec, Gleevec or STI-571; chemically designated as 4-[(4-methyl-1-piperazinyl)methyl]-N-[4-methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-phenyl]benzamide methanesulfonate) or a pharmaceutically acceptable salt thereof.

Another aspect of the invention is the use of imatinib, or a pharmaceutical acceptable salt thereof to treat T-ALL. Preferably, said T-ALL is characterized by a NUP214-ABL1 fusion.

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Still another aspect of the invention is the use of imatinib, or a pharmaceutical acceptable salt thereof to treat a cancer that is characterized by a NUP214-ABL1 fusion. Preferably, said cancer is a leukemia, even more preferably, said leukemia is selected from the group consisting of B-ALL, precursor B-LL and *BCR-ABL1* negative myeloproliferative diseases.

Specific aspects of the invention include:

- an isolated NUP214-ABL1 fusion gene comprising parts of the *NUP214* gene and *ABL1* gene, wherein said parts are due to breakpoints occurring in introns 23 to 34 of *NUP214* and intron 1 of *ABL1*;

- a mammalian episomal structure comprising a NUP214-ABL1 fusion gene as described herein;

- a method to detect a NUP214-ABL1 fusion gene as described herein or a mammalian episomal structure as described herein, comprising: obtaining cells; and determining the presence of said fusion gene or episomal structure in said cells; and

- use of imatinib, or a pharmaceutically acceptable salt thereof, to treat leukemia characterized by the presence of a NUP214-ABL1 fusion gene as described herein or of an episomal structure as described herein.

BRIEF DESCRIPTION OF THE FIGURES

Fig. 1 *FISH and array-CGH mapping of the amplified region on 9q34.*

a FISH results for patient 3. Extrachromosomal amplification of *ABL1* identified with the *BCR-ABL1* LSI probe (Vysis) in interphases and 1 metaphase. Normal chromosomes 22 (*BCR*) and 1 normal chromosome 9 are indicated. The other chromosome 9 carries a deletion of *ASS*, *FUBP3* and *ABL1*. Nine to 15 extra copies of *ABL1* are observed. Amplification of 3' *ABL1*, *LAMC3* and *NUP214* are also demonstrated, while *ASS*, *FUBP3*, 5' *ABL1* and *VAV2* are not amplified. *CDKN2A* shows heterozygous deletion. *b* Array-CGH results, showing signals for 138 clones from chromosome 9, confirm the amplification of *ABL1* and *LAMC3*. *NTNG1* is not amplified and 5' *ABL1* is only amplified in patient 1. The array does not contain a probe at the *NUP214* locus. Clone 1491 2 contains CDKN2A. *c* Location of the probes used for FISH and array-CGH and their location along chromosome 9 (based on ensembl data). black boxes represent genes, grey boxes represent BACs.

Fig. 2 *Detection of the NUP214-ABL1 fusion transcript by RT-PCR and Northern blot.*

a Detection of different NUP214-ABL1 fusion transcripts in 5 T-ALL patients with *ABL1* amplification (patients 1-4 and 6), and absence of this fusion in T-ALL patients lacking *ABL1* amplification (patients na1-na3). *NUP214-ABL1* fusion transcripts were also detected in 5 of 85 additional T-ALL patients screened by RT-PCR (patients 7-11). *b* Detection of different *NUP214-ABL1* fusion transcripts in 4 T-ALL cell lines by RT-PCR. *c* Detection of aberrant *ABL1* transcripts in the cell lines PEER, ALL-SIL and BE-13 by Northern blot. *d* Sequence of the detected *NUP214-ABL1* transcripts showing the different variants. All fusions are in-frame (sequence translated with the 1 letter amino acid abbreviations).

Fig. 3 *Schematic representation of the amplified region and the NUP214-ABL1 fusion protein.*

a Detailed scheme of the amplified region with the genes, and BAC clones indicated. *b* Schematic representation of the NUP214 and ABL1 proteins with the most important domains indicated. Two main NUP214-ABL1 fusion proteins are generated: a shorter fusion (239 kDa), detected only in 1 patient, and longer fusions (310 to 333 kDa), detected in the majority of patients. All fusions contain the predicted coiled-coil domains of NUP214 and the SH3, SH2 and tyrosine kinase domains of ABL1.

Fig. 4 *Characterization of the NUP214-ABL1 fusion protein.*

a Using anti-ABL1 and anti-NUP214 antibodies, the NUP214-ABL1 fusion protein could be detected in the cell lines ALL-SIL, BE-13 and PEER, but not in the *NUP214-ABL1* negative cell line LOUCY. NUP214-ABL1 was phosphorylated in the 3 cell lines, and phosphorylation of ABL1 was also detected in ALL-SIL. Phosphorylation of NUP214-ABL1 correlated with phosphorylation of CRKL. *b* Imatinib treatment of ALL-SIL results in a dose dependant decrease in phosphorylation of NUP214-ABL1 and CRKL. *c* Imatinib treatment has a dose dependent inhibitory effect on the growth of ALL-SIL cell line. As expected, no effect was observed on the LOUCY cell line, also indicating that the inhibitory effect observed with ALL-SIL is not due to a general toxic effect of imatinib on T-cells. *d* Imatinib treatment of primary bone marrow cells from patient 4 results in decreased phosphorylation of CRKL, demonstrating an inhibitory effect of imatinib on NUP214-ABL1 in primary leukemic cells from this patient.

Fig. 5. *The extrachromosomal elements (episomes) are not detected by standard cytogenetic analysis.*

FISH results with the LSI BCR-ABL1 probe (Vysis) for patient 3, showing hybridization of the *ABL1* probe (red) on one chromosome 9 (the other chromosome 9 has a deletion of *ABL1* and is thus not detected) and on multiple extrachromosomal elements (a), which are not visible by inverted DAPI staining (b). The *BCR* probe (green) hybridizes on the 2 normal chromosomes 22 (a,c).

EXAMPLES

Materials and methods to the examples

Patients

We selected retrospectively 90 cases of T-ALL with fixed cells available for the initial FISH screening. An additional patient (patient 6) was later added to the study, and a set of 85 T-ALL patients form the Dana Farber Cancer Institute (Boston, MA, USA) was screened for the presence of the *NUP214-ABL1* fusion by RT-PCR. The clinical diagnosis, morphology and immunophenotypic data were reviewed. This study was approved by the Ethical Committee of the Medical Faculty of the Leuven University.

Cytogenetics and FISH

Cytogenetic studies were performed on bone marrow or blood cells using direct or short-term cultures without mitogens and R-banding. Karyotypes were described according to the International System for Human Cytogenetic Nomenclature (ISCN) (Mitelman, 1995). In cases without bone marrow invasion, lymph node and/or pleural effusions were karyotyped. FISH

was performed on stored fixed cells suspension originally used for karyotyping, as described (Dierlamm *et al.*, 1996). We were able to hybridize successfully the same metaphases up to 3 times. Initial screening was done using the LSI BCR-ABL ES (Vysis, Downers Grove Il.) translocation probe. On average 10 metaphases and 200 nuclei were scored. Cases showing aberrant hybridization signals were further investigated using a panel of BAC probes mapping at 9q34 from 125.7 to 134.7 Mb according to ensembl (a joint project between European Bioinformatics Institute (EBI), an outstation of the European Molecular Biology Laboratory (EMBL), and the Wellcome Trust Sanger Institute (WTSI); both institutes are located on the Wellcome Trust Genome Campus in Hixton, south of the city of Cambridge, United Kingdom). FISH probes are listed in figure 1 and table 1. BACs were obtained from the Roswell Park Cancer Institute RPCI11 library (BACPAC Resource Center (BPRC), the Children's Hospital Oakland Research Institute, Oakland, California, USA).

Micro-array CGH

Array-CGH was performed using Code Linked Slides (Amersham Pharmacia Biotech, Piscataway, NJ) containing the 3527 BAC clones from the Wellcome Trust Sanger Institute 1 Mb Clone Set, a gift from Dr. N.P. Carter (Fiegler *et al.*, 2003). BAC DNA was amplified by "degenerate oligo nucleotide primed-PCR" (DOP-PCR) (Fiegler *et al.*, 2003). Aminolinked PCR products were spotted with a concentration of 200 ng/µl on the slides by use of a Molecular Dynamics Generation III printer (Amersham). The clones were printed in two replicates at different positions on the array. Test and reference genomic DNA was labeled by random prime labeling (Bioprime DNA Labelling System, Invitrogen, Carlsbad, CA) using Cy3 and Cy5 labeled dCTP's (Amersham Pharmacia Biotech). Probe preparation and pre-blocking of the slide was performed as described (Fiegler *et al.* 2003). Hybridization was performed for 48 hours under a coverslip in a humid chamber saturated with 20% formamide and 2x SSC. Post hybridization washes, and image and data analysis was performed according to standard conditions. Spot intensities were corrected for the local background. Only spots with signal intensities of Cy5 and Cy3 twofold

above background signal intensities were retained. For each clone a ratio of Cy5 over Cy3 fluorescent intensity was calculated. Normalization of the data was achieved by dividing the fluorescent intensity ratio at each spot by the mean of all ratios of the autosomes. Two values of the duplicate clones were averaged and a $\log_2$ value was calculated. If the variation among the two intensity ratios was larger than 10%, the datapoint was eliminated from the analysis. Log_2 ratios between -0.2 and 0.2 were accepted to be normal. Ratios below or above this normal range were interpreted respectively as due to a clone deletion or duplication. If the $\log_2$ ratio was above 1, then the clone was considered amplified.

Cell culture and Westem blotting

Bone marrow cells were incubated in RPMI-1640 supplemented with 10% FCS for a period of 2 hours in the presence of different concentrations of imatinib. Cells were lysed in 1.5x sample buffer, separated using SDS-PAGE and transferred to PVDF membranes. T-ALL cell lines

were cultured in RPMI-1640 supplemented with 20 % FCS. For dosis response curves, 3 x 10^5 cells / ml were grown in 24-well plates with different concentrations of imatinib, and viable cell number was determined at the beginning and after 24 and 48 hours of incubation. The percentage of viable cells relative to the control (no imatinib) was calculated at each time point for 3 independent wells. For Western blotting, the cell lines were cultured in the presence of different concentrations of imatinib for 2 hours, pelleted and lysed in cold lysis buffer containing 1 mM $NaVO_4$, and protease inhibitors. The proteins were separated on NuPAGE* Tris-Acetate gels (Invitrogen) and transferred to PVDF membranes. Antibodies used were: anti-phospho-ABL, anti-ABL, anti-phospho-CRKL and anti-CRKL (Cell Signaling); anti-mouse-PO and anti-rabbit-PO (Amersham Pharmacia Biotech). Anti-NUP214 antibody directed against the C-terminal part of NUP214 (Fornerod *et al.* 1995), was kindly provided by G. Grosveld (St Jude Children's Research Hospital, Memphis).

PCR

Rapid amplification of cDNA ends (RACE) was performed as described previously (Cools *et al.*, 1999). In short, cDNA synthesis was performed with ABL1-R1 (5'-gcgtgatgtagttgcttg), followed by PCR with the adaptor primers (Cools *et al.*, 1995) and the nested *ABL1* primers, ABL1-R2 (5'-acaccattccccattgtgattat) and ABL1-R3 (5'-ccggagcttttcacctttagtta). PCR products were cloned and sequenced. Direct RT-PCR to screen for the presence of *NUP214-ABL1* transcripts was performed using NUP20 (exon 20, 5'-aatccttgcccaaagtaccag), NUP28 (exon 28, 5'-tcacaccaacaccgtcttct), NUP29 (exon 29, 5'-agggaggctctgtctttggt), NUP31 (exon 31, 5'-agaggggaggtttcttcagt), combined with respectively ABL1-R2 and ABL1-R3. All PCR products were sequenced.

Southern and Northern blotting

For Southern blotting, 8 µg of DNA was digested with BamHI, size-fractionated on a 0.7% agarose gel, and transferred to Hybond*-N+ membranes (AP Biotech). The probe contained exon 2 – 8 of mouse Abl1 and was mixed with a probe for the TCRD gene on 14q11 (internal control). The hybridization signals were quantified by densitometric analysis using a Phosphor Imager and Image Quant*3.0 Software (Molecular dynamics, Sunnyvale, CA).

For Northern blotting, 10 µg of total RNA was fractionated on a 1 % agarose gel, and transferred to Hybond-N+ membranes (AP Biotech). The probe was a 300 bp PCR product of *ABL1*.

Example 1: *ABL1* is involved in T-cell acute lymphoblastic leukemia

The Philadelphia translocation, encoding the *BCR-ABL1* (*BCR-ABL*) fusion gene, is typically found in chronic myeloid leukemia (CML) and precursor B-cell acute lymphoblastic leukemia

*Trade-mark

(B-ALL), but is exceptionally rare in T-ALL (Pui *et al.*, 2004; de Klein *et al.*, 1982; de Klein *et al.*, 1986). To study the potential involvement of *ABL1* gene rearrangements in T-cell malignancies, we screened 90 T-ALL cases by fluorescence *in situ* hybridization (FISH), using *BCR* and *ABL1* probes. No *BCR-ABL1* fusion signals were observed, confirming the low frequency of this rearrangement in T-ALL. However, we observed marked amplification (> 10 signals per nucleus) of *ABL1* in 6 of 91 T-ALL patients (figure 1a, table 1, table 2, patients 1-6). Remarkably, the additional *ABL1* signals were extrachromosomal. Extrachromosomal amplification of oncogenes has been previously observed on double minute chromosomes (dmin) (Hahn, 1993), which are visible by standard cytogenetics, or on cytogenetically invisible units, referred to as episomes (Maurer *et al.*, 1987). In our cases, no dmin were visible by G or R banding, so *ABL1* amplification most likely occurred on episomes.

Detailed FISH mapping of the episomes confirmed that they contained *ABL1*, *LAMC3* and *NUP214* (*CAN*), 3 genes localized within a 500 kb region on chromosome region 9q34 (figure 1a, 1c). Probes containing the *ASS, FUBP2* and *VAV2* genes did not hybridize to the episomes (figure 1a, 1c). In addition, the 5' end of *ABL1* could not be detected on the episomes in 4 of the 6 cases, delineating the proximal breakpoint in the first intron of *ABL1* and the telomeric breakpoint between *NUP214* and *VAV2*. Micro-array-based comparative genomic hybridization (array-CGH), using a 1 Mb Bacterial artificial chromosome (BAC) array (Fiegler *et al.*, 2003), confirmed the amplification of *ABL1* and *LAMC3* in the 4 cases that could be analyzed by this method (figure 1b, 1c). BAC-57C19 sequences (5' *ABL1*) were only amplified in patient 1, confirming the FISH findings (figure 1b, table 1). BAC-5N16 sequences were not amplified in any of the 4 patients, further delimiting the amplified region to a maximal size of 1 Mb (figure 1b, 1c). In addition, array-CGH also showed deletion of BAC-149I2, containing the tumor suppressor gene *CDKN2A,* in patients 2-4, an observation that was also confirmed by FISH (figure 1a, 1b, table 1). In patients 1-3, amplification of *ABL1* was also confirmed by Southern blot.

Example 2: *ABL1* is fused to *NUP214*

The presence of a breakpoint within intron 1 of the *ABL1* gene, and the selective absence of the 5'-end of *ABL1* in the amplicon, suggested that *ABL1* might be involved in the generation of a fusion gene. To test this, we performed RACE-PCR on *ABL1* transcripts from patient 4. Sequencing of the PCR products revealed an in-frame fusion between exon 31 of *NUP214* and exon 2 of *ABL1* (figure 2a, 2d). These results are compatible with a model in which the genomic region between *ABL1* and *NUP214* was circularized, generating a *NUP214-ABL1* fusion gene (figure 3a). The copy number of the episome then increased due to unequal segregation during cell division. In patient 3, episome formation may have originated from a deletion, as one chromosome 9 carries a deletion of a region slightly larger than the amplified

region (figure 1a, 1b, table 1). Similar deletions associated with amplification of *MYC* on dmin or episomes were reported (Carroll *et al.*, 1988).

We next performed RT-PCR to detect *NUP214-ABL1* fusion transcripts in the 5 patients with *ABL1* amplification for which cDNA was available. All cases expressed a *NUP214-ABL1* fusion transcript that was absent in T-ALL cases without *ABL1* amplification (figure 2a). To confirm these findings, an additional 85 T-ALL patients were screened by RT-PCR. The *NUP214-ABL1* fusion transcript was detected in 5 additional patients (patients 7-11) (figure 2a). We next tested human T-ALL cell lines for the presence of the fusion. The *NUP214-ABL1* transcript was detected in 3 of 22 independent T-ALL cell lines: ALL-SIL, PEER, and TALL-104, and in BE-13, a tetraploid subline of PEER (figure 2b, table 3). The presence of an aberrant *ABL1* transcript was also confirmed by Northern blot in the cell lines (figure 2c). Taken together, these data indicate that approximately 6% of T-ALL patients harbor a cryptic fusion of *NUP214* to *ABL1*.

Variants of the *NUP214-ABL1* fusion gene were observed among T-ALL cases due to different breakpoints in *NUP214* (ranging from introns 23 to 34) (figure 2d). All breakpoints in *ABL1* occurred in intron 1, with exon 2 of *ABL* thus present in all fusion variants. The exon usage of *ABL1* was thus invariant and coincided with the *ABL1* breakpoint observed in Phildephila positive CML and B-ALL. The *NUP214-ABL1* fusion mRNAs are predicted to encode proteins of 2210 to 3175 amino acids with molecular weights of approximately 239 to 333 kDa (figure 3b).

NUP214 is an FXFG repeat-containing protein that is a component of the nuclear pore complex, that mediates nucleocytoplasmic transport (Kraemer *et al.*, 1994). The *NUP214* gene is widely expressed and is involved in the pathogenesis of acute myeloid leukemia associated with the t(6;9)(p23;q34) / *DEK-NUP214* fusion (Von Lindern *et al.*, 1992). However, in the DEK-NUP214 fusion protein, the C-terminal region of NUP214 (encoded by exons 18-36) is present, whereas the predicted NUP214-ABL1 fusions retain the N-terminal region of NUP214 (encoded by variants ranging between exons 1-23 to exons 1-34) that includes the predicted coiled-coil domains of NUP214 that may serve as oligomerization motifs (figure 3b).

Example 3: NUP214-ABL1 acts as a constitutively activated tyrosine kinase

ABL1 is an ubiquitously expressed cytoplasmic tyrosine kinase that is fused to BCR in CML and precursor B-ALL cases with the t(9;22)(q34;q11) (de Klein *et al.*, 1982; de Klein *et al.*, 1986), and to ETV6 in leukemias with the t(9;12)(q34;p13) (Golub *et al.*, 1996). Similar to BCR-ABL1 and ETV6-ABL1 fusion proteins, the NUP214-ABL1 fusions contain the SH3, SH2 and kinase domains of ABL1 (figure 3b), suggesting that NUP214-ABL1 acts as a constitutively activated tyrosine kinase. This was assessed by analysis of the tyrosine phosphorylation state of NUP214-ABL1 and CRKL, a direct target of the ABL1 kinase (Oda *et al.* 1994). The

NUP214-ABL1 fusion protein was detected in the cell lines PEER, BE-13, and ALL-SIL, using antibodies directed against ABL1 or NUP214 (figure 4a). In addition, NUP214-ABL1 and CRKL were phosphorylated in the NUP214-ABL1 expressing cell lines, whereas CRKL was unphosphorylated in the NUP214-ABL1 negative cell line LOUCY (figure 4a). Addition of imatinib, a selective inhibitor of ABL1 kinase activity (Capdeville *et al.*, 2002), decreased phosphorylation of both NUP214-ABL1 and CRKL (figure 4b), and inhibited the proliferation of ALL-SIL (figure 4c). Phosphorylation of CRKL was also inhibited by imatinib in primary bone marrow cells from patient 4 (figure 4d). These results indicate that NUP214-ABL1 is a constitutively activated tyrosine kinase that activates similar pathways as BCR-ABL1, and is sensitive to inhibition with imatinib.

While constitutively activated tyrosine kinases are sufficient to induce myeloproliferative disease, they require the cooperative effect of other mutations to induce acute leukemia (Kelly and Gilliand, 2002). In agreement with this, we identified additional mutations in the cells expressing the *NUP214-ABL1* fusion. Deletion of the *CDKN2A/B* (p16/p15) tumor suppressor genes was detected in 7 of 8 evaluable cases, and deletion of 12p, a region that may harbor a tumor suppressor gene (Hoornaert *et al.*, 2003) was observed in the one patient without *CDKN2A/B* deletion. Molecular screening for known T-ALL oncogenes revealed the mutually exclusive overexpression of *TLX1 (HOX11)* and *TLX3* (*HOX11L2*) in respectively 4 and 5 patients (Table 2). Similar findings were observed in the T-ALL cell lines, where expression of *TLX1* and *NKX2-5* was reported for ALL-SIL and PEER, respectively (table 3) (Nagel *et al.*, 2003). These data provide genetic support for a multi-step pathogenesis of T-ALL: deletion of a tumor suppressor gene (*CDKN2A/B* or a putative chromosome 12p tumor suppressor), deregulated expression of a transcription factor (*HOX11, HOX11L2*), and expression of a constitutively activated tyrosine kinase (*NUP214-ABL1*).

Our results identify the episomal fusion of *NUP214* to *ABL1* as a novel mechanism for the generation of a fusion gene. This gene rearrangement is cryptic by conventional cytogenetics (figure 5), but readily detected by FISH using a commercially available *ABL1* probe. Indeed, Barber et al. recently reported *ABL1* amplification in a subset of T-ALL patients, although the molecular and physiologic consequences of amplification were not elucidated (Barber *et al.*, 2004). FISH with the *ABL1* probes is distinctive, and appears to be pathognomonic for the presence of the *NUP214-ABL1* fusion in T-ALL. *ABL1* hybridization on 9q34 is observed as expected, and at multiple extra-chromosomal sites in metaphases, as well as multiple signals in the majority of interphase cells. This study also demonstrates the power of high resolution array-CGH for the detection of acquired genetic unbalances in cancer cells. We show that besides cryptic deletions (Cools *et al.*, 2003) also cryptic amplifications can generate novel fusion genes, suggesting that genome-wide screens for deletions and amplifications in a broad spectrum of hematologic malignancies and solid tumors may reveal more of these aberrations.

It will be interesting to investigate if this fusion also occurs in T-ALL as a result of t(9;9)(q34;q34), or in other hematological malignancies, in particular precursor B-ALL and *BCR-ABL1* negative myeloproliferative diseases. Survival data of our 11 patients indicates a rather aggressive course of disease in the 4 adults (2 early relapses and 2 toxic deaths) (table 2). The finding that NUP214-ABL1 is sensitive to the tyrosine kinase inhibitor imatinib suggests novel therapeutic approaches to improve the outcome and potentially decrease treatment related morbidity in the subset of T-ALL cases that express the *NUP214-ABL1* fusion gene.

TABLES

Table 1: FISH results in 6 patients with abnormal *ABL1* hybridization pattern

patient	LSI *BCR-ABL1*		number of signals observed with the used BAC probes								
			373J8	202H3	618A2/17L7	57C19	83J21	143H20	544A12	153P4	83N9
	nuclei	metaphases[1]	(*NIBL*)	(*GPR107*)	(ASS/ *FUBP3*)	(*5'ABL1*)	(*3'ABL1*)	(*LAMC3*)	(*NUP214*)	(*VAV2*)	(*LHX3*)
1 [bm]	m (82 %)	3 / 9	2	2	2	m	m	m	m	2	2
2 [pb]	m (75 %)	12 / 19	2	2	2	2	m	m	m	2	2
3 [bm]	m (76 %)	14 / 20	2	1	1	1	m	m	m	2	2
4 [bm]	m (23 %)	16 / 40	2	2	2	2	m	m	m	3 (20%)	2
5 [bm]	m (6 %)	13 / 30	2	2	2	m	m	m	m	2	2
6 [pe]	m (96 %)	9 / 10	na	na	2	2	m	na	m	2	na
6 [bm]	m (16 %)	1 / 1									

All BAC probes are derived from the RPCI-11 library, the location of most of the BACs/genes is shown in figure 1c. m, multiple (> 10); [bm]on bone marrow; [pe]on pleural effusion; [pb]on peripheral blood; na, not analyzed; [1]number of abnormal metaphases / total examined.

Table 2: Characteristics of 11 T-ALL patients with *ABL1* amplification and/or *NUP214-ABL1* fusion

patient	sex/ age	WBC ($x10^9$/L)	% blast (BM)	immuno-phenotype[a]	Karyotype[b]	FISH results		molecular results		response to therapy / outcome	OS (months)
						CDKN2A[c]	*ABL1*	*NUP214-ABL1*	other[d]		
1	M / 52	134	96	cortical	46,XY,del(12)(p13) [4]/ 46,XY [1]	+/+	ampl.	+	*HOX11*	CR, early relapse	7
2	M / 3	162	95	cortical	46,XY [12]	-/-	ampl.	+	*HOX11*	CR	57+
3	M / 23	81	92	cortical	48,XY,t(3;11)(p12;p15), t(7;10)(q35;q24), t(8;10)(q21;q21),+11,+12 [5] / 46,XY [4]	-/+	ampl.	+	*HOX11*	CR, alloBMT / toxic death	10
4	M / 7	196	88	mature	46, XY [33]	-/+	ampl.	+	*HOX11L2*	CR	40+
5	M / 6	52	82	pre-T	47,XY,del(6)(q21),+8 [12] / 46,XY [4]	+/+	ampl.	na	na	CR, early relapse	14
6	M / 25	8	40[e]	mature[f]	46,add(X)(p22)Y, t(8;22)(p22;q12), del(13)(q14q22)	-/+	ampl.	+	*HOX11L2*	early toxic death	0.5
7	F / ped	na	96	cortical	near tetraploid with del(11)(q23)	-/-	na	+	*HOX11L2*	CR	194+
8	F / ped	na	86	mature[f]	na	-/-	na	+		CR, early relapse	7
9	M / ped	na	na	na	na	na	na	+	*HOX11L2*	CR	14+
10	M / ped	na	na	na	na	-/-	na	+	*HOX11L2*	CR	176+
11	F / 31	na	75	pre-T	46,XX [20]	na	na	+	*HOX11*	early toxic death	1

na, not available; OS, overall survival in months; CR, complete remission; ped, pediatric;
[a] Following EGIL classification
[b] Karyotype obtained from bone marrow culture at diagnosis, except for patient 2 where peripheral blood was analyzed.
[c] +/+: no deletion, -/+ hemizygote deletion, -/- homozygote deletion.
[d] Molecular screening for *BCR-ABL1*, *SIL-TAL1* and *ETV6-AML1* transcripts, *TLX1*, *TLX3* and *MLL* rearrangement (Southern Blot).
[e] 40 % blasts in bone marrow and 77 % in pleural effusion
[f] Mature T phenotype with aberrant expression of CD13.

Table 3:

	cell line	*NUP214-ABL1*	*TLX1*	*TLX3*	*NKX2-5*
1	ALL-SIL	+	+	–	–
2	BE-13*	+	–	–	+
3	CCRF-CEM	–	–	–	+
4	DND-41	–	n.d.	n.d.	n.d.
5	DU-528	–	–	–	–
6	HPB-ALL	–	–	+	–
7	HSB-2	–	–	–	–
8	JURKAT	–	–	–	–
9	KARPAS-45	–	–	–	–
10	KE-37	–	–	–	–
11	KOPT-6	–	n.d.	n.d.	n.d.
12	LOUCY	–	–	–	–
13	MHH-TALL1	–	–	–	–
14	MHH-TALL2	–	–	–	–
15	MOLT-3	–	n.d.	n.d.	n.d.
16	MOLT-4	–	–	–	–
17	MOLT-13§	–	–	–	–
18	MOLT-14§	–	n.d.	n.d.	n.d.
19	MOLT-16#	–	–	–	–
20	MOLT-17#	–	n.d.	n.d.	n.d.
21	P12-ICHIKAWA	–	–	–	–
22	PEER*	+	+	–	+
23	RPMI-8402	–	–	–	–
24	SUP-T1	–	–	–	–
25	SUP-T11	–	n.d.	n.d.	n.d.
26	TALL-104	+	–	–	–

List of the 26 cell lines screened by RT-PCR for presence of the *NUP214-ABL1* fusion. This collection contains 22 independent T-cell lines, and 4 sister cell lines (indicated by *, §,#). Expression data on *TLX1*, *TLX3*, and *NKX2-5* were reported by Nagel et al. (2003).

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SEQUENCE LISTING IN ELECTRONIC FORM

In accordance with Section 111(1) of the Patent Rules, this description contains a sequence listing in electronic form in ASCII text format (file: 29775-64 SEQ 12-03-2012 v1.txt).

A copy of the sequence listing in electronic form is available from the Canadian Intellectual Property Office.

The sequences in the sequence listing in electronic form are reproduced in the following table.

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29775-64

CLAIMS:

1. An isolated NUP214-ABL1 fusion gene comprising parts of the *NUP214* gene and *ABL1* gene, wherein said parts are due to breakpoints occurring in introns 23 to 34 of *NUP214* and intron 1 of *ABL1*.

2. An isolated NUP214-ABL1 fusion gene according to claim 1, wherein said fusion gene encodes a tyrosine kinase.

3. An isolated NUP214-ABL1 fusion gene according to claim 2, wherein said tyrosine kinase is constitutively activated.

4. A mammalian episomal structure comprising a NUP214-ABL1 fusion gene according to any one of claims 1 to 3.

5. A method to detect a NUP214-ABL1 fusion gene according to any one of claims 1 to 3 or a mammalian episomal structure according to claim 4, comprising:

- obtaining cells; and

- determining the presence of said fusion gene or episomal structure in said cells.

6. The method of claim 5, wherein said method comprises FISH analysis.

7. The method of claim 5, wherein said method comprises micro-array-CGH.

8. The method of claim 5, wherein said method comprises PCR.

9. The method of claim 5, wherein the presence of the fusion gene or episomal structure is indicative of leukemia.

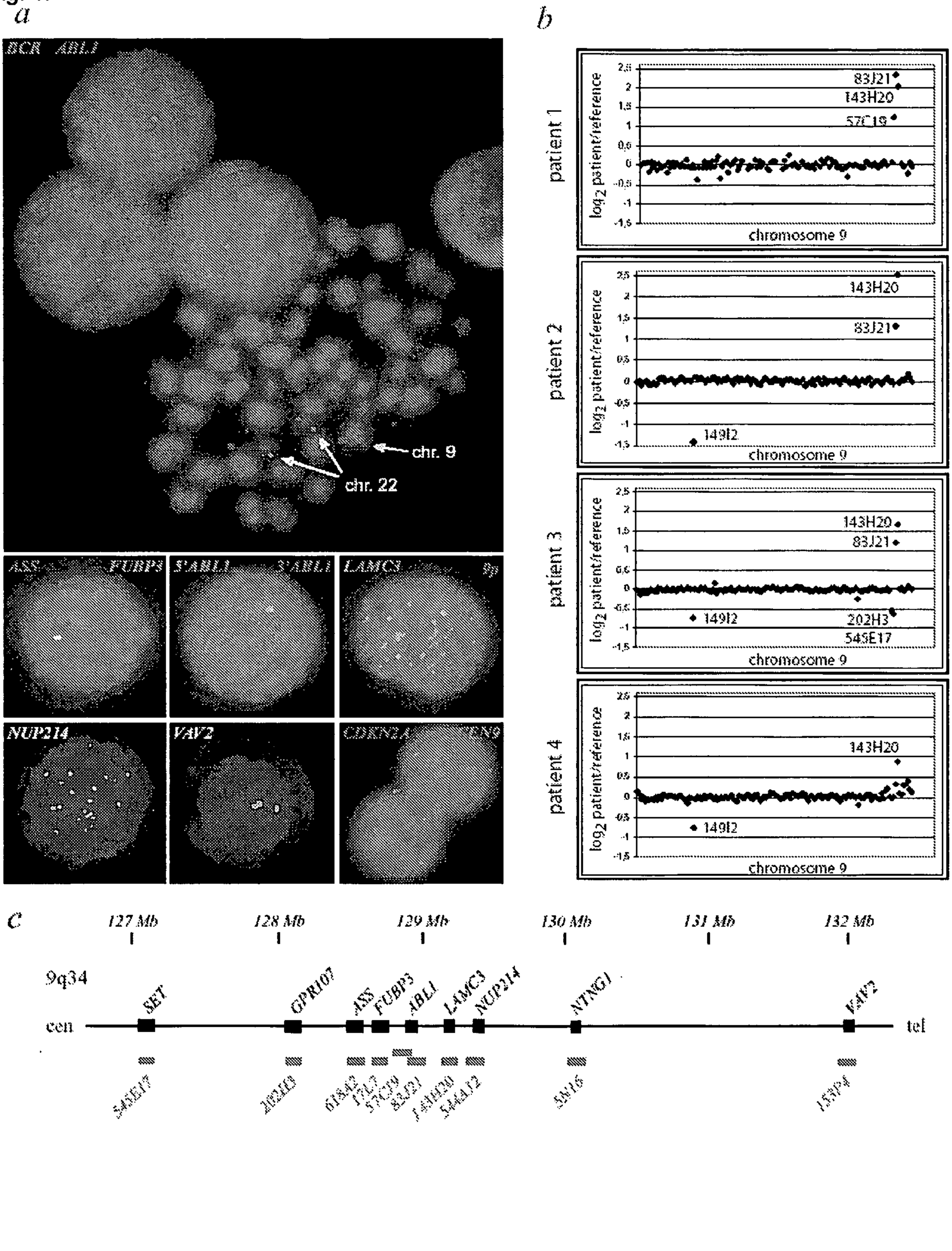
10. The method of claim 9, wherein the leukemia is T-ALL.

29775-64

11. Use of imatinib, or a pharmaceutically acceptable salt thereof, to treat leukemia characterized by the presence of a NUP214-ABL1 fusion gene according to any one of claims 1 to 3 or of an episomal structure according to claim 4.

12. The use according to claim 11, wherein the leukemia is T-ALL.

Fig. 1: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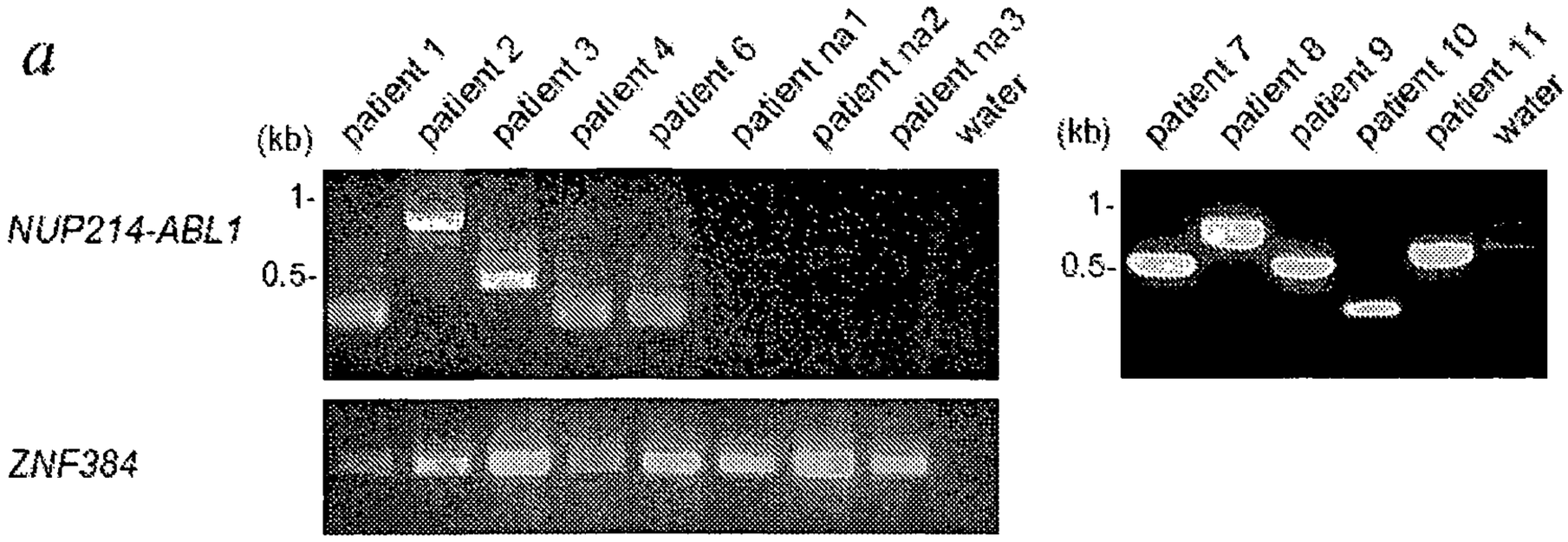

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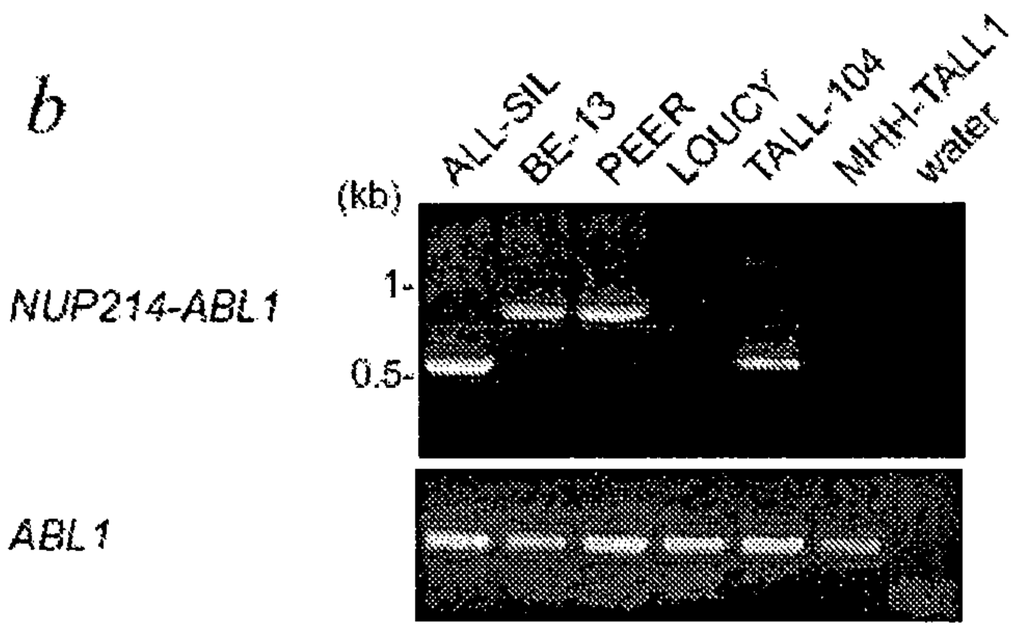

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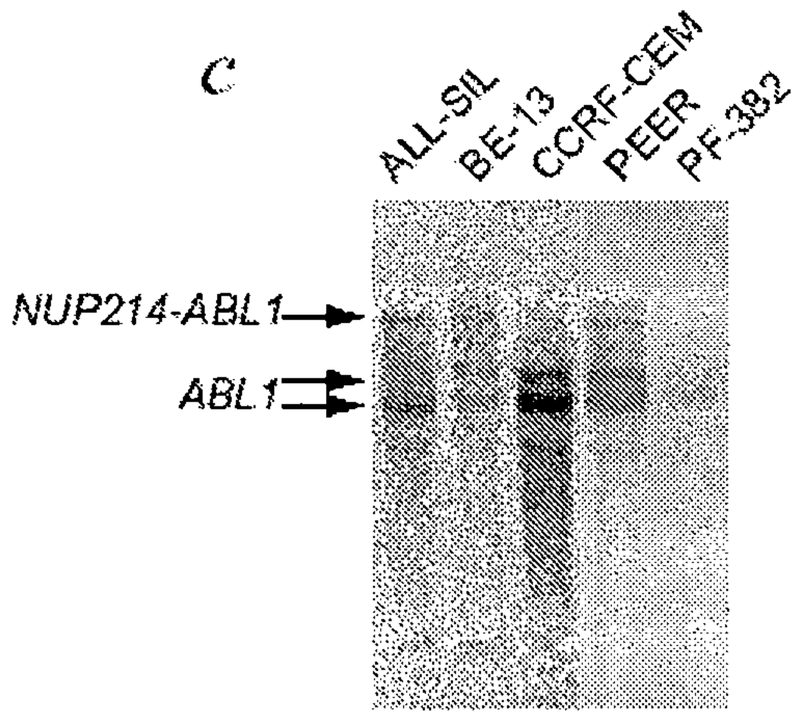

d

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patient 10	*NUP214 (exon 29)*	***ABL1 (exon 2)***
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patients 1, 4, 6, 7, 9, 11	*NUP214 (exon 31)*	***ABL1 (exon 2)***
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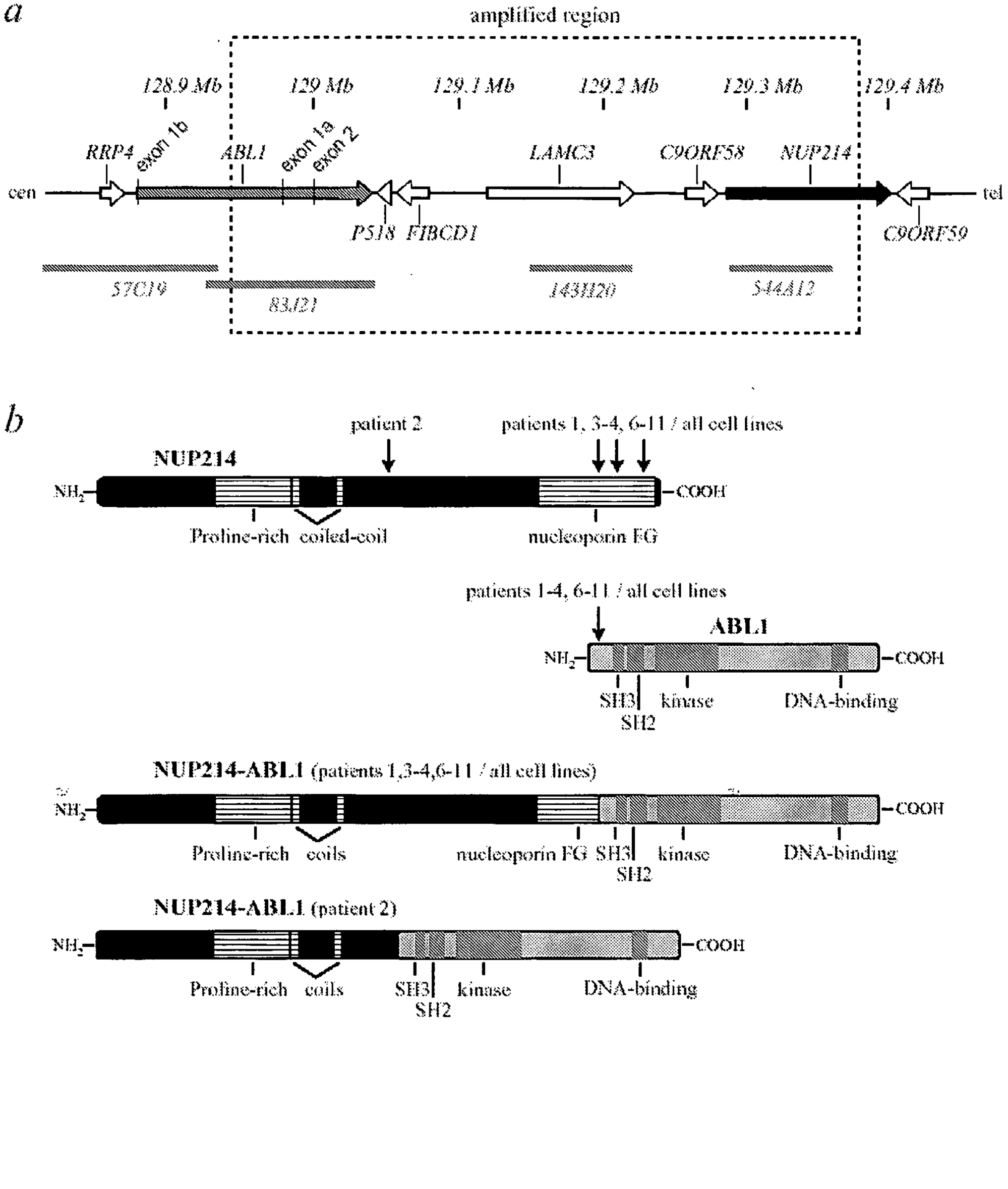
Fig. 3: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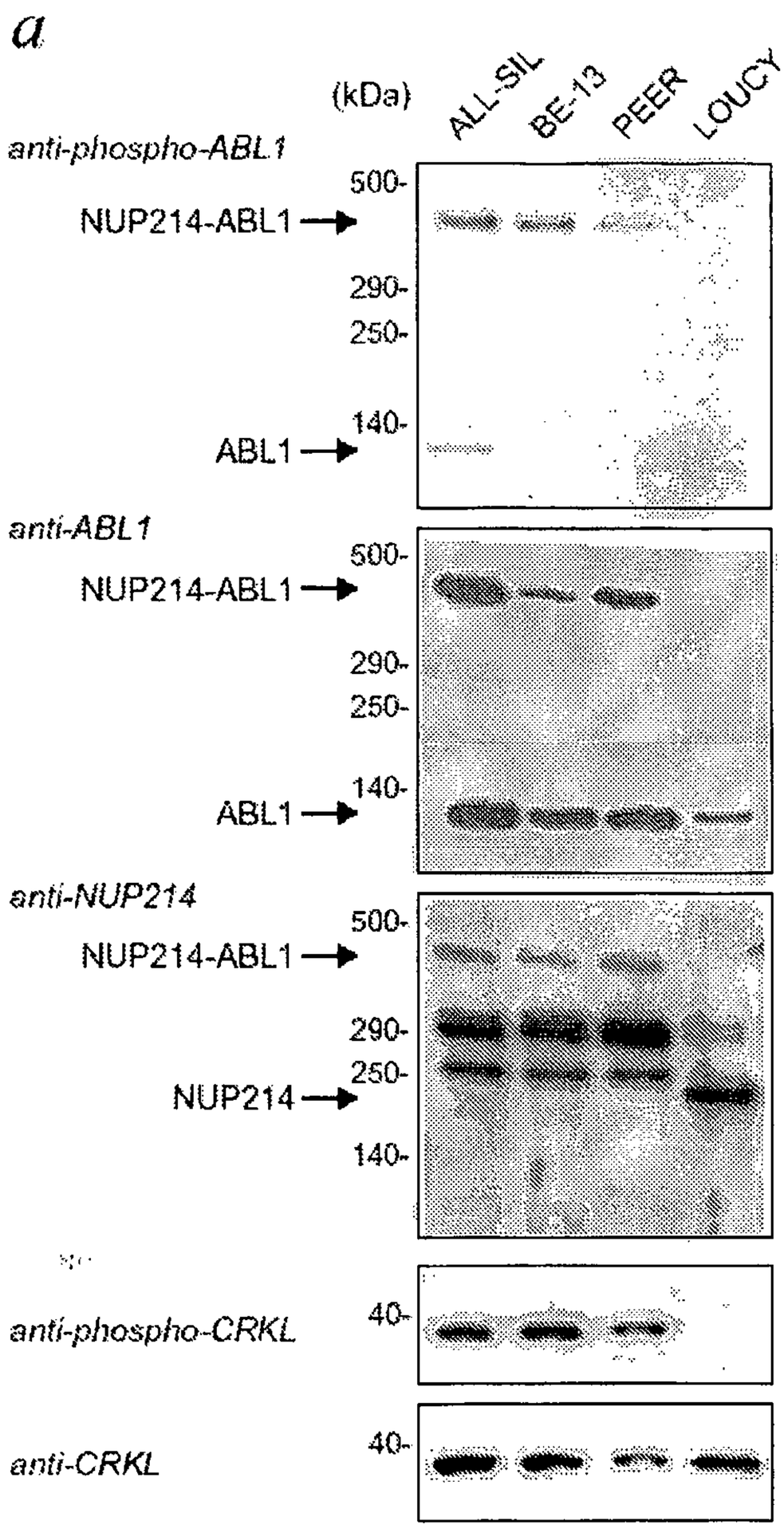
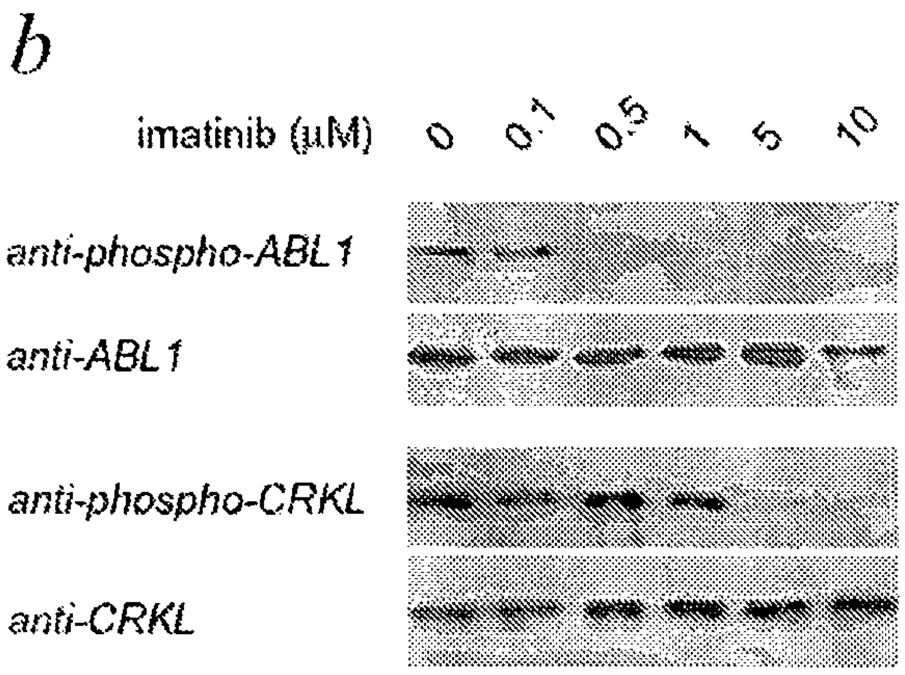
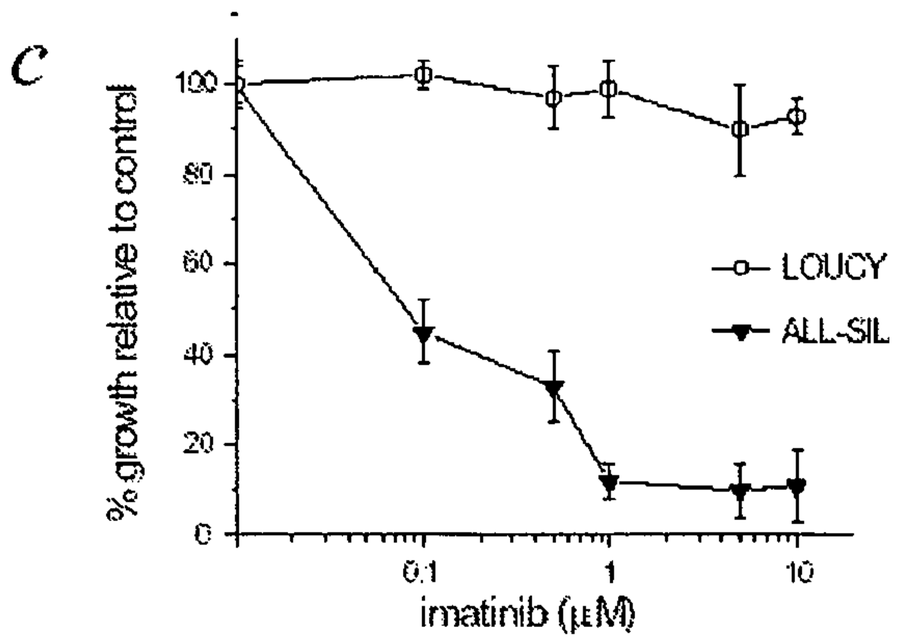
C9ORF59
57C19
83J21
143J20
544A12
b
patient 2
patients 1, 3-4, 6-11 / all cell lines
NUP214
NH2
COOH
Proline-rich
coiled-coil
nucleoporin FG
patients 1-4, 6-11 / all cell lines
ABL1
SH3
SH2
kinase
DNA-binding
NUP214-ABL1 (patients 1,3-4,6-11 / all cell lines)
Proline-rich
coils
nucleoporin FG
NUP214-ABL1 (patient 2)

Fig. 4:


a
(kDa)
ALL-SIL
BE-13
PEER
LOUCY
anti-phospho-ABL1
500-
NUP214-ABL1
290-
250-
140-
ABL1
anti-ABL1
500-
NUP214-ABL1
290-
250-
140-
ABL1
anti-NUP214
500-
NUP214-ABL1
290-
250-
NUP214
140-
anti-phospho-CRKL
40-
anti-CRKL
40-


b
imatinib (µM)
0
0.1
0.5
1
5
10
anti-phospho-ABL1
anti-ABL1
anti-phospho-CRKL
anti-CRKL


c
% growth relative to control
100
80
60
40
20
0
0.1
1
10
imatinib (µM)
LOUCY
ALL-SIL

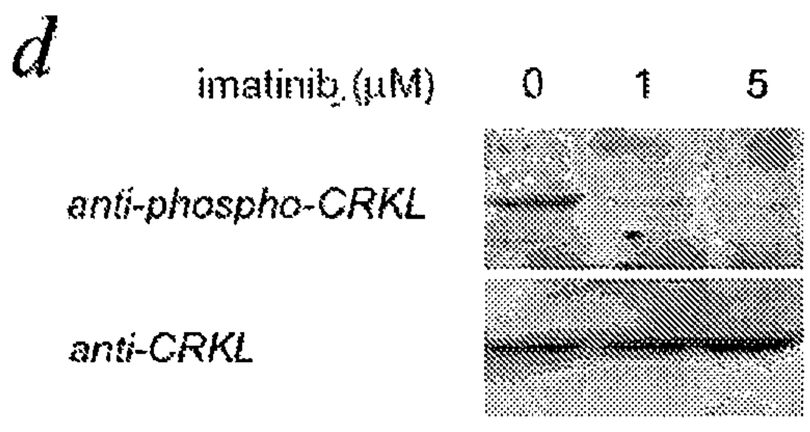

d
imatinib (µM)
0
1
5
anti-phospho-CRKL
anti-CRKL

Fig. 5: