Id4 is deregulated by a t(6;14)(p22;q32) chromosomal translocation in a B-cell lineage acute lymphoblastic leukemia

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Background and Objectives. Chromosome translocations resulting in gene overexpression are commonly associated with lymphoid neoplasia. Enhancer elements of the immunoglobulin or T-cell receptor (TCR) loci are abnormally located in the vicinity of the entire coding sequences of genes which exert an influence on the normal maturation and differentiation program of lymphoid cells.

Design and Methods. A patient who presented with a B-cell lineage acute lymphoblastic leukemia had a t(6;14)(p22;q32). Cytogenetic and molecular findings confirmed the involvement of IgH. Molecular cloning of the breakpoint revealed that this was located near the coding sequence of the *Id4* gene, a helix-loop-helix (HLH) inhibitor protein. *Alu*-repeated sequences at the 6p22 end flanked a short stretch of 10 bases shared by the 6p22 and 14q32 ends, suggesting that a deletion or a looping-Alu mediated mispairing mechanism may lead to this chromosome translocation.

Results. Northern blot and real-time polymerase chain reaction analyses showed that the *Id4* mRNA was abnormally overexpressed in this case. Only the two smaller *Id4* mRNA products were detected (1.6 and 1.1 kb). Immunohistochemical analysis of Id4 protein was also assayed in a series of hematologic malignancies. Marked overexpression was found in two cases of T-cell prolymphocytic leukemias and in four B-cell lineage acute lymphoblastic leukemia including one case with the t(8;14) and another case with a p53 mutation.

Interpretation and Conclusions. The *Id4* gene may behave as an oncogene in some human leukemias, perhaps through its capacity to sequester specific B-cell transcription factors. A genetic recombination between Alu-repeated sequences may not be the exclusive mechanism of generating pathogenic chromosomal translocations.

Key words: Id4, leukemia, chromosomal translocations.

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Chromosomal translocations are very common molecular abnormalities in hematologic malignancies. The cloning of translocation breakpoints has proven to be one of the most efficient ways of identifying new genes that are involved in regulating cell growth and inducing malignant transformation.

In some lymphomas and leukemias, the juxtaposition of promoter/enhancer elements from immune receptor genes with the intact coding region of other genes (*c-myc*, *bcl2*, *bcl6*) determines the neoplastic phenotype by alterating cell cycle progression, braking the physiologic process of apoptosis or modifying lineage-specific transcription machinery.[1]

Cytogenetic abnormalities of chromosome 6 that have been reported in hematologic malignancies include different chromosomal translocations, amplifications and deletions. t(6;14)(p25;q32) causes a deregulation of MUM1/IRF4 in patients with mature plasma cell malignancies (multiple myeloma).[2] Recently, Sonoki *et al.*[3] identified cyclin *D3* as the target gene in some low grade B-cell chronic lymphoproliferative disorders with the t(6;14)(p21.1;q32.3). In this report, the involvement of *Id4*, a helix-loop-helix (HLH) inhibitor, was demonstrated in one case of B-cell lineage acute lymphoblastic leukemia with the t(6;14)(p22;q32). This finding widens the repertoire of chromosome 6 translocations with pathogenic relevance in lymphoid neoplasms. Furthermore, abnormal overexpression of an inhibitory transcription factor may be a recurrent mechanism of neoplastic transformation.

Design and Methods

Patient's clinical characteristics

A 31-year old woman was admitted to the Hospital de la Santa Creu i Sant Pau in May 2001 with peripheral blood blastosis. She had a past medical history of long-lasting hirsutism, amenorrhea, acanthosis nigricans and obesity. Endocrine studies revealed an increase in thyroid-stimulating hormone with an associated decrease in sex hormone-binding globulin.

Two months before admission, the patient had complained of easy bruising associated with thrombocytopenia and was treated with corticosteroids.

Peripheral blood counts at diagnosis were: hemoglobin 120 g/L, leukocytes 21520×10^3/L with 41% blasts, platelets 24×10^9/L. A bone marrow aspirate revealed a a 60% infiltration of lymphoblasts with a high nuclear/cytoplasm ratio, convoluted nuclear contour,

marked nucleoli in some elements and the absence of vacuolization or granules in the cytoplasm. Immunophenotypic, cytogenetic and molecular studies were performed in the same sample. According to the FAB classification, a B-cell lineage ALL-L2 was diagnosed and the patient was treated with an induction cycle of chemotherapy consisting of vincristine, L-asparaginase, prednisone and cyclophosphamide. The percentage of bone marrow blasts on day 14 was 8%. By the end of this phase the patient had achieved a complete remission. In May 2001 she was admitted for consolidation chemotherapy with high doses of methotrexate. She developed a major neurologic complication with cerebellar symptoms which evolved into respiratory arrest, and she died 5 months after the leukemia had been diagnosed. Morphologic and immunophenotypic studies performed at that time (minimal residual disease assessment) were negative.

Flow cytometry immunophenotyping

The immunophenotype of the leukemic blasts was established using triple combinations of fluorochrome-conjugated monoclonal antibodies analyzed by conventional flow cytometry methods, as reported elsewhere.[4]

Cytogenetics and fluorescence in situ hybridization studies

Cytogenetic analysis was performed on 24-hour unstimulated cultures of bone marrow cells. Chromosomes were G-banded with Wright's stain and the karyotype described according to the International System for Human Cytogenetic Nomenclature.[5] Fluorescence *in situ* hybridization (FISH) studies were carried out on destained G-banding meta phases using a commercial dual color IgH break-apart probe (Vysis, Downers Grove, IL, USA) following the recommendations of the manufacturer. Data were collected on a fluorescence microscope Leica-DM equipped with a cooled CCD camera Sensys (Photometrics, Tucson, AZ,USA) run by PathVysion software (Vysis, Stuttgart, Germany).

Long-distance inverse polymerase chain reaction

DNA was digested with proteinase K, extracted using the *salting out* method and precipitated with ethanol. Long-distance inverse polymerase chain reaction (LDI-PCR) and DNA sequencing of the IgHJ rearrangement were performed as described elsewhere, with minor modifications.[6]

Briefly, high molecular weight DNA was completely digested with HindIII enzyme. Purified DNA was then ligated at 15°C overnight with 5U of T4 DNA ligase (New England Biolabs, MA, USA). The ligated DNA was purified and eluted in a final volume of 40 µL. Primers employed in the following amplification process were:

JE6 CCCACAGGCAGTAGCAGAAAACAA;
J6I:TCTGGGCTCGAGTCGACGCAGAAAACAAAG GCCCTAGAGGG;
JHE:TGGGATGCGTGGCTTCTGCT;
JHI:GCCCTTGTTAATGGACTTGGAGGA.

A hot start protocol of amplification was used. After PCR amplification the products were purified and run on 1% agarose. Bands corresponding to the size expected from Southern analysis were excised from the gel purified with Qiaex II (Qiagen, Hilden, Germany) and ligated into TOPO-TA (Invitrogen, Groningen, NL) vector.

Southern and Northern blots

Southern blot. Five micrograms of DNA were digested using Eco RI and Hind III (New England Biolabs, MA, USA) restriction enzymes. DNA was separated on a 0.7% agarose gel and transferred to nylon membranes (Amersham Ltd, Buckinghamshire, UK), which were hybridized with the IgHJ6 probe labeled with ^{32}P-dCTP by the random primer extension method. Filters were washed in 0.2×SSC(NaCl/Na citrate)/0.5% sodium dodecyl sulphate (SDS) for 2 hours at 65°C and then autoradiographed using intensifying screens.[7]

For *Northern blotting* an *Id4* cDNA probe was used. The NorthernMax kit (Ambion, Austin, TX, USA) formaldehyde system was employed following the recommendations of the manufacturer.

Sequencing and alignment

Plasmids containing the correct size insert were sequenced with an ABIPRISM 310 (Applied Biosystems, Foster City, CA, USA) automated sequencer employing both forward and reverse sequencing primers. Sequences were compared with those in the Human Genome Database using the BLAST program via on line resources made available by the NCBI home page.

Real-time PCR

Id4 mRNA transcript levels were determined by means of the pre-developed Assay on demand® with the ABIPRISM 7700 Sequence Detector (Applied Biosystems). A validation experiment was performed in parallel with *abl* transcript levels, demonstrating that PCR efficiency of target (*Id4*) and reference (*abl*) were equal. This previous assay allowed us to apply the $\Delta\Delta$ C_T method of quantification (PE Applied Biosystems User Bulletin 2; ABI PRISM 7700 Sequence Detection System, 1997). Three normal bone marrow samples, 20 B-cell lineage ALL and 2 peripheral blood samples were used as controls. *Id4* mRNA values were referred to those observed in normal bone marrow samples and reactive lymph nodes.

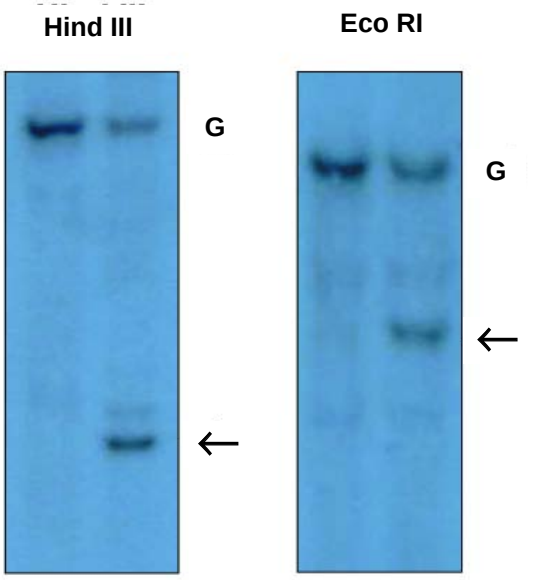

Figure 1. Southern blot analysis of IgH genes using the IgHJ6 probe showed a single rearranged band (arrows) on *Hind III* (3.8 kb) and *Eco RI* (9 kb) digests. G: germ line bands.

Immunohistochemistry

Formalin-fixed, paraffin-embedded tissue biopsies from 11 patients diagnosed with different types of hematologic malignancies were cut into 5 µm sections. After deparaffinization and rehydration, heat-induced antigen retrieval was performed by immersing sections in 0.01M sodium citrate buffer (pH 6.0) and incubating in an autoclave (121° C for 10 min). Endogenous peroxidase activity was blocked with 0.3% H_2O_2. Rabbit anti-human Id4 (Santa Cruz Biotechnology; 1:50) was incubated for 30 min. and visualized with the EnVision system (Dako, Glostrup, Denmark) using 3-3' diaminobenzidine tetrahydrochloride as the chromogen. Sections were counterstained with hematoxylin. The techniques were performed using a Dako Autostainer.

Results

Immunophenotypic, cytogenetic and molecular findings

The leukemic cells had the following immunophenotype: cytoplasmic CD79a, nuclear Tdt^+, surface HLA-Dr, CD10, CD20, CD19, CD22, CD38, CD99 and FMC7 all positive, being negative for myeloperoxidase, lysozyme, lactoferrin, CD15, CD34, CD2, CD3, CD33, CD7, CD117, CD13, CD66, CD123, CD56, CD64, CD36, CD4, CD11b, CD5, CD16 and surface immunoglobulin. CD45 was dimly positive. The complete karyotype was: 46XX,-1,-3,del(4)(q?), add(5)(q31), t(6;14)(p22;q32), +3 mar [11]/46XX [14]. Southern blot analysis revealed a single rearranged band on EcoRI and HindIII digests (Figure 1).

Molecular cloning of t(6;14)(p22;q32)

FISH analysis with the dual color IgH break-apart probe revealed a split of the original fluorescence signal to the short arm of chromosome 6 (green) and the long arm of chromosome 14 (red) confirming the translocation t(6;14)(p22;q32) (Figure 2).

Based on the cytogenetic and molecular results, an inverse-PCR approach was employed in order to characterize the rearrangement. J6E/JHE J6I/JHI combination primers were used in a nested PCR protocol following the procedure established by Willis *et al.*[6] A single 1.2 kb band was successfully amplified and the product was cloned on a TOPO-TA cloning vector (Invitrogen). The insert was sequenced using both forward and reverse sequencing primers. Sequence results matched perfectly with the following two clones: gi 3676217(47830-47876) for the 6p22 end and gi 1770449 for the 14q32 end (91677-91653)(Figure 3).

Id4 is abnormally overexpressed as result of the chromosomal translocation

Northern blotting revealed a marked overexpression of the *Id4* gene in the patient's sample when compared with expression in normal bone marrow samples. In these normal cases no signal was detected. Two bands corresponding to 1.6 kb and 1.1 kb were demonstrated, suggesting that *Id4* was present in two splice variants. Real-time PCR results showed a marked increase in the Ct values when comparing the cDNA obtained from the case with the t(6;14) and the normal bone marrow controls, peripheral blood and B-cell lineage ALL (20 samples) (Figure 4).

Id4 is also overexpressed in other hematologic neoplasms

Id4 expression was also investigated using a polyclonal antibody in a series of biopsies from patients with hematologic malignancies (11 cases) and 4 controls (two central nervous system biopsies and two normal bone marrow samples). *Id4* overexpression was detected in 6 cases: 4 biopsies from patients with B-cell lineage ALL (Figure 5) and 2 samples from patients with T-cell prolymphocytic leukemias. The percentage of positivity ranged between 30 to 100% of cells. A predominant nuclear staining pattern was observed in the positive cases. In one case there was an associated p53 mutation and another case had a t(8;14) involving the *c-myc* locus (Table 1).

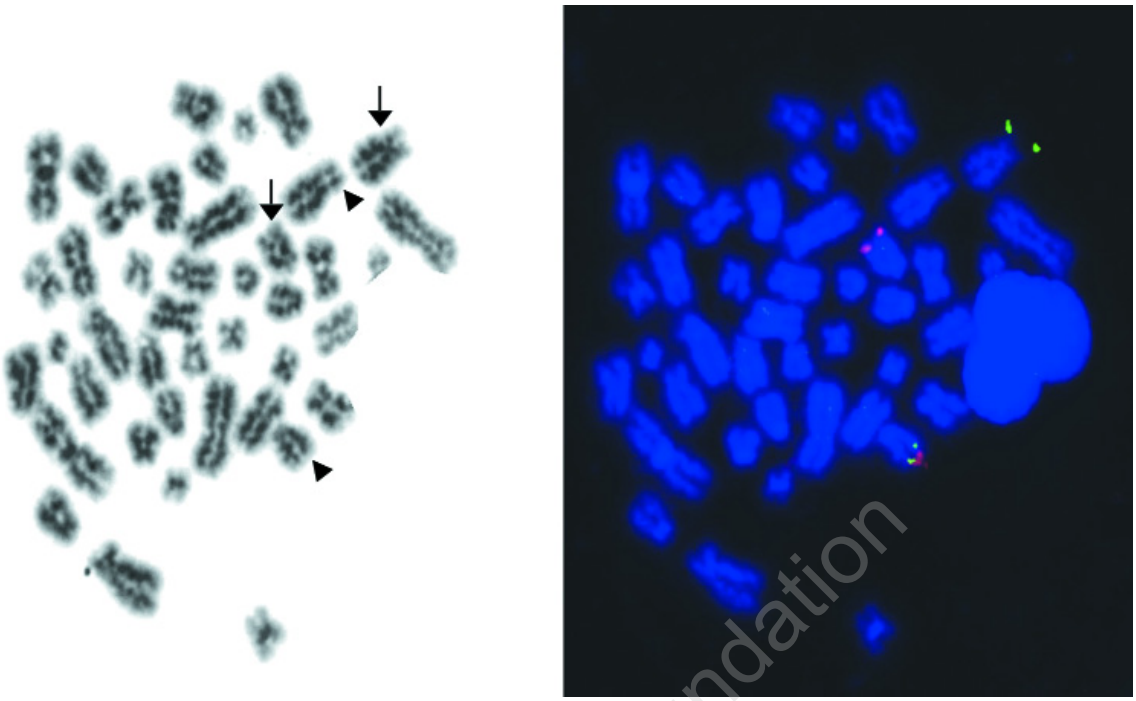

Figure 2. Karyotypic findings: conventional chromosome G-banding (left) and FISH studies (right) showed a t(6;14)(p22;q32). Short arms of chromosome 6: green signal. Long arms of chromosome 24: red signal.

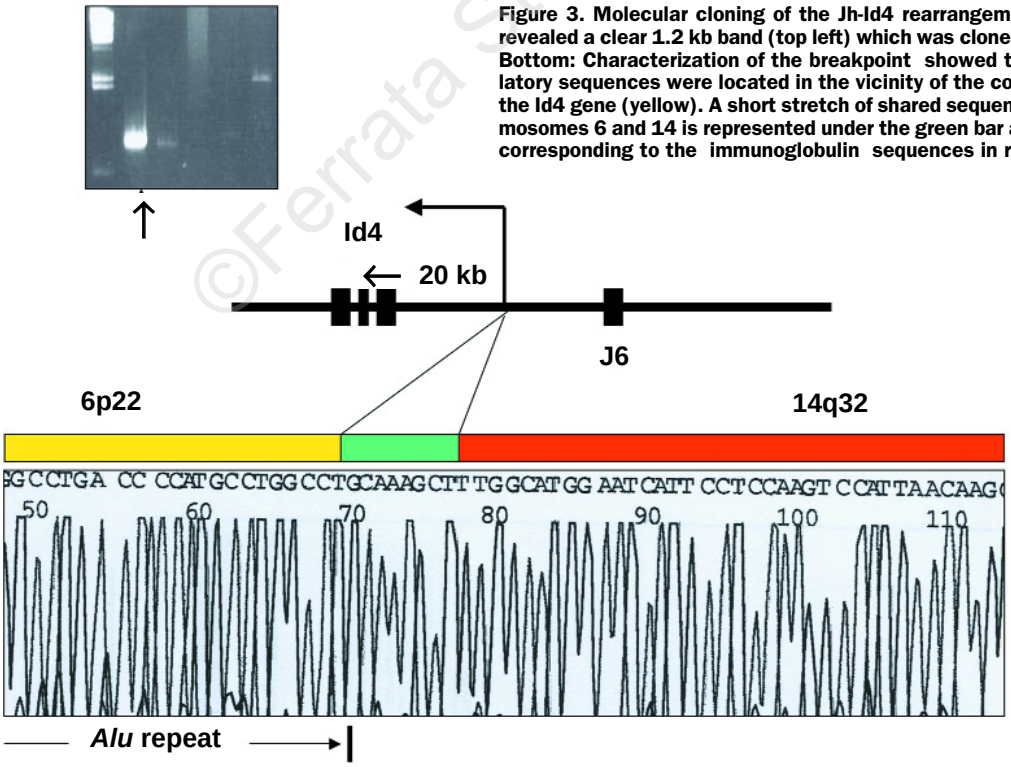

Figure 3. Molecular cloning of the Jh-Id4 rearrangement. Inverse PCR revealed a clear 1.2 kb band (top left) which was cloned and sequenced. Bottom: Characterization of the breakpoint showed that the IgH regulatory sequences were located in the vicinity of the coding sequence of the Id4 gene (yellow). A short stretch of shared sequence between chromosomes 6 and 14 is represented under the green bar and the sequence corresponding to the immunoglobulin sequences in red.

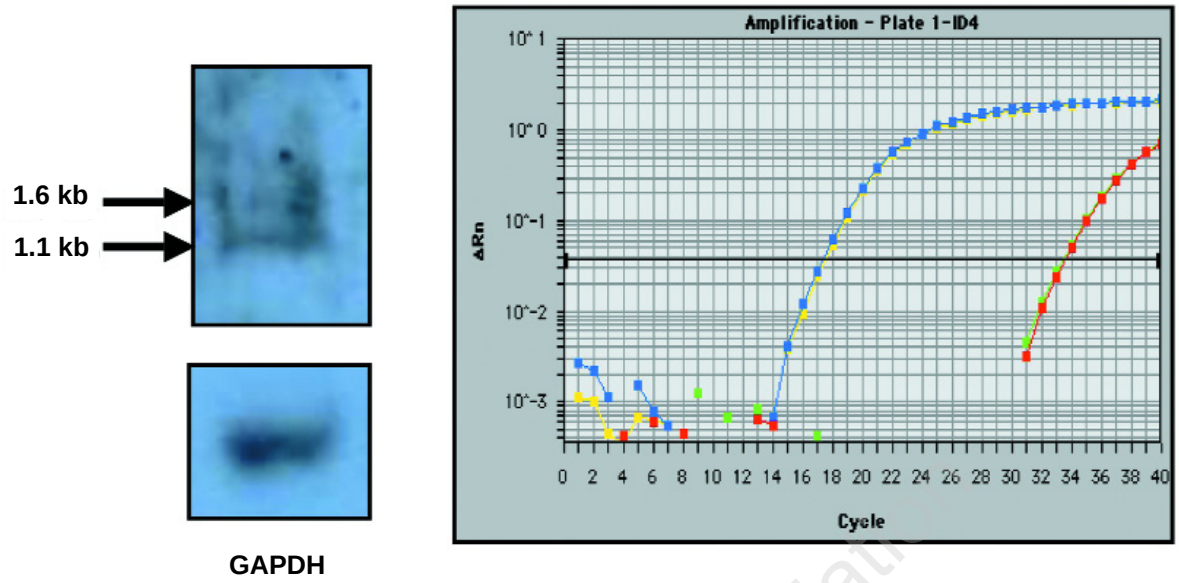

Figure 4. Id4 mRNA assessment by Northern blot (left) and real time PCR (right). Two overexpressed Id4 bands (1.6 and 1.1 kb) were identified in the sample obtained from the patient with the t(6;14)(p22;q32). The Ct value for the patient was 18 whereas the Ct in another case of acute leukemia (B-ALL) was 34.

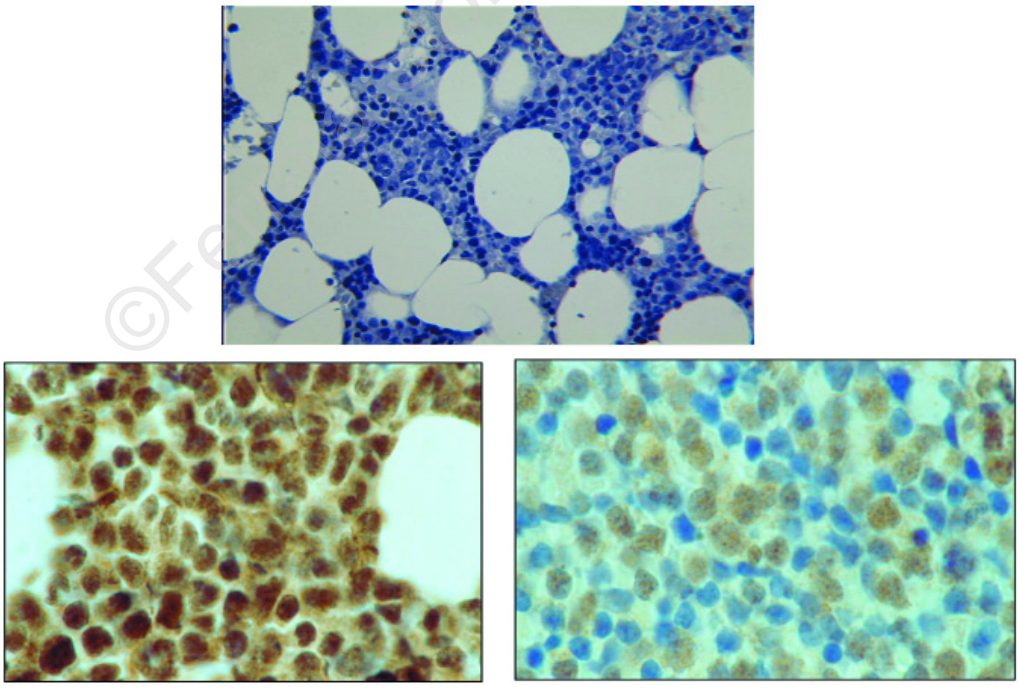

Figure 5. Top: Normal bone marrow showed no positive cells reacting with the Id4 antibody. Original magnification x160. Bone marrow biopsies stained with an Id4 antibody showed strong nuclear positivity in a B-ALL case (bottom left) and moderate positivity (bottom right) in another B-ALL case. Top: Normal bone marrow showed no Id4 positive cells. Original magnification x400.

Table 1. Id4 expression in hematologic neoplasms.

Pts.	*Diagnosis*	*Sample*	*Cytogenetics/ molecular genetics*	*Id4*
UPN 1	B-ALL	Soft tissue mass	p53 mutation	+++
UPN 2	Biphenotypic AL	Testicular relapse	MLL rearrangement	–
UPN 3	T-ALL	Testicular relapse	TCRb rearrangement	–
UPN 4	AML	Bone marrow	Monosomy 7	–
UPN 5	B-ALL	Bone marrow	46 XY 12p-	+++
UPN 6	Biphenotypic AL	Bone marrow	Normal karyotype	–
UPN 7	T-ALL	Spleen	TCRβ rearrangement	–
UPN 8	T-PLL	Bone marrow	TCRβ rearrangement	+++
UPN 9	B-ALL	Bone marrow	t(8;14)(q24;q32)	+
UPN 10	T-PLL	Bone marrow	TCRβ rearrangement	+++
UPN 11	B-ALL	Bone marrow	Hyperdiploidy	++

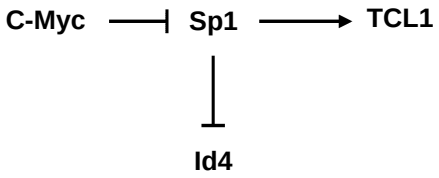

Figure 6. Regulatory pathways controlling Id4 expression. c-Myc inhibits Sp1 transcriptional activity which in turn represses Id4 expression. Furthermore, Sp1 transactivates TCL1 oncogene which becomes deregulated in T-PLL. Sp1 suppression may lead to abnormal Id4 expression without a t(6;14) chromosomal translocation.

Discussion

This is the first description of a chromosomal translocation involving Id4, a bHLH inhibitor in human lymphoid neoplasia. Four *Id* genes have been cloned from the mouse — *Id1*, *Id2*, *Id3* and Id4 — and human homologs have been mapped at chromosomes 20q11, 2p25, 1p36 and 6p22. These genes share considerable homology.[8-16] The class V HLH proteins, which include Id4, act as dominant negative regulators by sequestering the ubiquitously expressed bHLH transcription factors and by preventing them from forming active heterodimers. Id proteins contain a highly conserved HLH motif but lack an adjacent basic region necessary for DNA binding. It has been found that the down-regulation of Id is necessary for differentiation to proceed in many cell lineages including those of the lymphohematopoietic system. Ectopic expression of *Id* genes, reported here, inhibits differentiation.[17–19] Two functional regions have been characterized in the Id4 promoter: a consensus E-box and a GA motif, which is the target of Sp1 and Sp3 for repressing Id4 expression.[20]

Overexpression of Id4, caused by enhancer activity of the Ig locus, leads to a functional haploinsufficiency of important transcription factors controlling B-cell development (E2A and Pax).[21,22] As has been previously demonstrated, E2A-deficient mice rapidly develop T-cell lymphomas and ectopic expression of Id2 (another member of the class V HLH proteins) in thymocytes leads to the rapid development of lymphomas. Loss of E2A homodimers through targeted gene disruption and ectopic expression of other bHLH proteins such as Lyl1 or Tal1 play a crucial role in the appearance of lymphoid leukemias.[8,9]

In B-ALL the normal function of E2A is truncated by chromosomal translocations associated with t(1;19) and t(17;19). In these models, the disruption of one E2A allele contributes to leukemogenesis. Interestingly, these leukemias show an immunophenotype very similar to that observed in the present case (cytoplasmic IgM^{+}, CD20^{++}).[23]

It has also been reported that Id2 can reverse the inhibition of cellular proliferation mediated by pRb, p16 and p21. Constitutive activation of the N-Myc-Id2 pathway plays a major part in the pathogenesis of human neuroblastoma.[24,25] This pathway operates through the primary amplification and/or overexpression of the *N-myc* oncogene. The final outcome is accumulation of Id2 protein to levels that are sufficient to inactivate pRB. A similar mechanism may act in some lymphoid leukemias. Furthermore, another Id protein, Id1, has a repressor capacity over p16/Ink4a and overexpression of Id proteins has been associated with colorectal adenocarcinoma with high p53 expression.[26]

Although Id4 deregulation by chromosomal translocation does not seem to be a common finding in ALL, Id4 overexpression may be present in some lymphoid leukemias as suggested by our immunohistochemical studies. Id4 may be overexpressed as a result of the release of the normal inhibitory signals provided by Sp1 and Sp3, which act on regulatory sequences of the Id4 promoter. Sp1 and Sp3 inhibition may also arise as a consequence of chromosomal translocations involving C-myc or TCL-1 in Burkitt's lymphoma and T-PLL, respectively.[20,27,28] It may be assumed that abnormal TCL1 expression observed in T-PLL supplies a negative signal over Sp transcription factors. This reg-

ulation process is depicted in Figure 6.

The analysis of the sequences in the genetic breakpoint of the case presented here provides information about the basic mechanisms of chromosomal translocations. Genetic recombination between intronic Alu repeated elements has been recognized as an important factor in MLL leukemias.[29,30] Alu repeats have long been recognized as a deletion hot spot in many genetic diseases. We postulate that the inadequate resolution, in a lymphoid precursor, of a deletion in the Alu sequence may underlie the abnormal recombination.

Further studies are warranted to shed light on the role of Id4[31] and other inhibitory bHLH transcription factors in human neoplasias.

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Pre-publication Report & Outcomes of Peer Review

Contributions

MB, JN: designed the study and wrote the draft; JS participated in discussing the results, particularly the clinical findings; RB, CP, XM-G: performed the immunohistologic studies and critically discussed the results. AA: responsible for the cytogenetic studies; AL, CE, MJC, MB: responsible for the molecular studies.

Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

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Manuscript processing

This manuscript was peer-reviewed by two external referees and by Professor Mario Cazzola, Editor-in-Chief. The final decision to accept this paper for publication was taken jointly by Professor Cazzola and the Editors. Manuscript received July 8, 2003; accepted July 18, 2003.

In the following paragraphs, Professor Cazzola summarizes the peer-review process and its outcomes.

What is already known on this topic

A few translocations involving chromosome 6 have been described in patients with lymphoid malignancies.

What this study adds

This study shows that Id4, a helix-loop-helix inhibitor, may behave as an oncogene in patients with lymphoid malignancy and chromosome 6 translocations.