Infant Acute Leukemias Show the Same Biased Distribution of *ALL1* Gene Breaks As Topoisomerase II Related Secondary Acute Leukemias

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Advances in Brief

Infant Acute Leukemias Show the Same Biased Distribution of *ALL1* Gene Breaks As Topoisomerase II Related Secondary Acute Leukemias[1]

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Abstract

The *ALL1* gene (also called *MLL, HRX,* or *Htrx1*) at the cytogenetic band 11q23 is consistently altered by chromosome rearrangements in acute leukemias (ALs) of early infancy, in ALs developed after exposure to topoisomerase (topo) II-inhibitory drugs, and in a small subset of *de novo* ALs in children and adults. Because exposure to natural or medicinal substances blocking topo II during pregnancy have been proposed as etiological agents for infant leukemia, we have compared the distribution of *ALL1* gene breakpoints in infant leukemias with an altered *ALL1* gene configuration to those in secondary leukemia associated with prior exposure to topo II targeting drugs and in reference to the major topo consensus binding site in exon 9. *ALL1* gene breakpoint distribution was determined by Southern blot hybridization and/or reverse transcription-PCR of the ALL1/AF4 fusion cDNA in 70 patients. Using restriction enzyme analysis, the 8.3-kb ALL1 breakpoint cluster region was divided in a centromeric portion of 3.5 kb (region A) and telomeric portion of a 4.8 kb (region B). ALL1 breakpoint were located in region A in 8 of 28 (28.5%) cases of infant ALs, 16 of 24 (66%) cases of *de novo* ALs, and 0 of 5 cases of therapy-related (TR) ALs. Conversely, ALL1 breakpoints in region B were detected in 20 of 28 (71.5%) cases of infant AL, 8 of 24 (33%) cases of *de novo* AL, and 5 of 5 (100%) cases of TR AL ($P = 0.002$). These results were confirmed by direct sequencing of the ALL1/AF4 fusion transcript in 30 cases (19 infants and 11 child and adult *de novo* cases). The analysis of ALL1/AF4 junction types showed that children and adults with *de novo* leukemia had ALL1 breakpoints in intron 6 (9 cases) or intron 7 (2 cases), whereas breakpoints in infant cases were mainly located in intron 8 (14 cases) and less frequently in intron 6 (4 cases) and intron 7 (1 case). The difference in ALL1 breakpoint location between infant and noninfant AL patients with ALL1/AF4 fusion was statistically significant ($P = 0.00005$).

These data demonstrated that infant and TR ALs share a similar biased clustering of *ALL1* gene breakpoints, which supports the possibility that topo II inhibitors may also operate *in utero* and play a crucial role in the etiology of infant leukemia.

Introduction

Chromosome translocations and resultant oncogene activation are common genetic events in the pathogenesis of leukemia. However, the molecular mechanism underlying reciprocal translocations leading to gene fusions is not yet clearly understood (1–3).

The *ALL1* gene (also called *MLL, HRX,* and *Htrx 1*) at the cytogenetic band 11q23 (4–6) is altered by promiscuous chromosome recombinations with a variety of partner genes in various subsets of ALs,[3] including a proportion of childhood and adult acute lymphoid or myeloid leukemia; secondary leukemias associated with prior exposure to drugs that target topo II (etoposide, teniposide, and anthracyclines); and, especially, infant leukemia (<12 months), in which the majority of cases have the *ALL1* gene fusions (7–13). Several DNA motifs implicated in DNA recombination mechanisms have been recently identified and localized within the ALL-1 bcr. These include: (*a*) recombinase signal sequences (heptamers and nonamers); (*b*) SARs; (*c*) high-affinity topo II consensus binding sites including a strong site in exon 9; and (*d*) Alu-sequences (13–15). By comparing ALL1 rearrangements in *de novo versus* TR ALs, Strissel-Broeker *et al.* (13) have reported statistically significant differences in breakpoint distribution between the two groups. In particular, they found that in TR ALs, breakpoints clustered in the telomeric portion of the ALL1 bcr, which is characterized by presence of SARs and high-affinity topo II binding sites, in contrast to cases of *de novo* ALs, which mostly had breaks in the centromeric or 5′ bcr.

On the basis of these observations, the authors suggested that translocation mechanisms in *de novo* and TR ALs might be different (13).

This conclusion has important implications for attempts to understand the etiology and pathogenesis of ALs in infants. Molecular analyses of *ALL1* gene rearrangements in infant twins showed that these genetic aberrations arise during fetal hemopoiesis *in utero* (15). Epidemiological evidence has also indicated that ceratin pregnancies are associated with increased risk of infant leukemia (16).

On the basis of an analogy with the involvement of the *ALL1* gene in TR secondary leukemias, it was suggested that the critical event(s) *in utero* might similarly involve exposure to topo II inhibitors (17). Several such natural and medicinal substances are available as candidate agents (17–20). If infant AL breakpoints were biased away from (centromeric to) the major topo II site in exon 9, this would make such an etiological model less likely. In the study of Strissel-Broeker *et al.* (13), only a small number (six) of the *de novo* cases were in fact infants (*i.e.*, 1 year of age or less). We have therefore compared *ALL1* gene breakpoint distribution in a larger group of infant leukemias with those *de novo* childhood or adult ALs and TR ALs.

Materials and Methods

Patient Samples. Seventy patients (ages, 1 month to 65 years) with *ALL1* gene rearrangement were included in this study. Of these, 33 cases (29 infants and 3 children) were referred to the Associazione Italiana Ematologia Oncologia Pediatrica, 15 patients with *de novo* ALs and 5 with TR ALs were seen at the Institute of Hematolgy of University "La Sapienza" of Rome, and the remaining 7 infants were referred to the Leukemia Research Fund Center as

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[3] The abbreviations used are: AL, acute leukemia; topo, topoisomerase; bcr, breakpoint cluster region; TR, therapy-related; SAR, scaffold attachment region; RT-PCR, reverse transcription PCR.

part of the United Kingdom National Case/Control Epidemiological Study of Childhood Cancer. Informed consent was obtained from parents and patients for both biological and treatment procedures. Diagnosis was established according to standard morphological, cytochemical, and immunological criteria. All patients were studied at the time of diagnosis. Mononuclear cells from bone marrow or peripheral blood samples obtained by centrifugation on a Ficoll-Hypaque density gradient were used for immunophenotypic and molecular studies.

DNA Analysis. High molecular weight DNA was extracted from mononuclear cells, digested to completion with various restriction enzymes (*Bam*HI, *Hin*dIII, *Bgl*II, and *Xba*I), sized fractioned by electrophoresis through a 0.8% agarose gel, and transferred onto nitrocellulose membranes. Filters were prehybridized for 16–24 h at 42°C in a solution containing 50% deionized formamide, 5× Denhardt's solution, and 100 μg/ml denatured salmon sperm DNA and then hybridized with probes previously labeled with ^{32}P by the random priming technique. To study the ALL1 genomic configuration we used the following three distinct probes: the B859 cDNA insert, which contains ALL1 exons 5–11 sequences (4); the B351 insert, which is a 351-bp cDNA insert obtained by *Hin*fI digestion of the B859 segment and contains the ALL1 exon 5 and 6 sequences; and the genomic Alu-free 0.4-kb FA4 insert (Ref. 21; Fig. 1*a*). Filters were washed twice for 30 min in 0.2× SSC and 0.1% SDS at 65°C and exposed with an intensifying screen at −70°C for 24–72 h.

RNA Preparation. Total RNA was extracted according to the method of Chomczynski and Sacchi (22). The quality of RNA was assessed on an ethidium bromide-stained 1% agarose gel containing 2.2 mol/liter formaldehyde.

ALL1-AF4 RT-PCR Amplification. *In vitro* reverse transcription of 1 μg of total RNA to cDNA was performed at 42°C for 20 min in a 20-μl reaction volume containing 2.5 units of cloned Moloney murine leukemia virus reverse transcriptase and random examers as primers, using a commercial kit (GeneAmp RNA PCR kit; Perkin-Elmer Corp., Norwalk, CT) according to the manufacturer's instructions. A volume of 5 μl was then diluted with 95 μl of a PCR mixture containing 1.5 mmol/liter $MgCl_2$, 50 mmol/liter KCl, 10 mmol/liter Tris-HCl (pH 8.3), 200 μmol/liter dNTPs, 2.5 units of Taq DNA polymerase (Perkin-Elmer Corp.) and 15 pmol of primers Ex 5 and AF4.1. After an initial denaturation at 94°C for 2 min, 30 cycles of amplification were performed on a DNA Thermal Cycler (Perkin-Elmer-Cetus). One cycle of denaturation, annealing, and extension consisted of 94°C for 1 min, 56°C for 1 min, and 72°C for 1 min, respectively. At the end, 1 μl of a 1:10 dilution of the first PCR product was used for a second round of amplification for 30 further cycles using the primers Ex 6 and AF4.1 (half-nested PCR). Finally, 1 of 10 of PCR products was run on a 2% agarose gel stained with ethidium bromide and visualized under a UV lamp. The sequences of primers used were as follows: Ex 5, 5′-GAG GAT CCT GCC CCA AAG AAA AG-3′ sense; Ex 6, 5′-CGC CCA AGT ATC CCT GTA AAA C-3′ sense; AF4.1, 5′-TGA GCT GAA GCT GGT CTT CGA GC-3′ antisense.

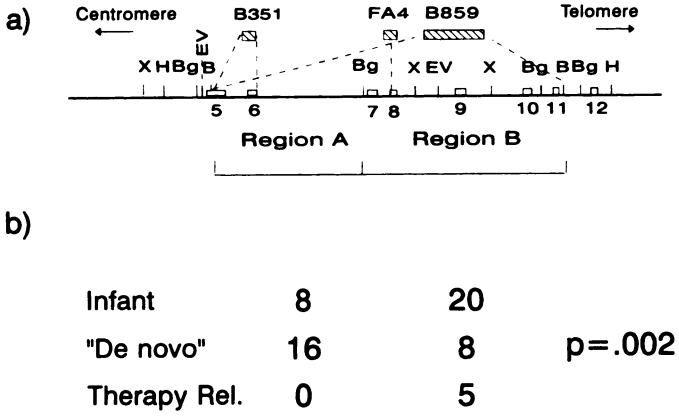

Fig. 1. *a*, genomic organization of the ALL1 bcr and location of the three probes used for breakpoint mapping. *Open numbered boxes*, ALL1 exons 5–12. *Regions A* and *B*, distinguished by a *Bgl*II site, represent two distinct centromeric and telomeric regions in which patient breaks were located. Restriction enzymes are indicated as follows: *X*, *Xba*I; *H*, *Hin*dIII; *Bg*, *Bgl*II; *EV*, *Eco*RV; *B*, *Bam*HI. *b*, location of ALL1 breakpoints in the three distinct patient categories.

Table 1 *Clinical and biological characteristics of the 70 patients with ALL1-positive ALs*

	Infant AL	*De novo* AL	TR AL
Total cases	36	29	5
Median age (range)	5 mo (0.1–12 mo)	39 yr (3–65 yr)	42 yr (31–48 yr)
Sex			
Male	11	13	0
Female	25	16	5
Phenotype[a]			
Early B[b]	24	12	
Pre-B	3	3	
M2		2	
M4–M5	5	12	5

[a] Early B, HLA-DR+/CD19+/CD10−; Pre-B, HLA-DR+/CD19+/CD10+.
[b] Available in 32 infants.

Amplification of the normal *ALL1* gene mRNA was performed with the same cDNA preparation and the same conditions used to identify the ALL1/AF4 junctions, using the following primers: Ex 6, 5′-CGC CCA AGT ATC CCT GTA AAA C-3′ sense; Ex 7: 5′CTT AAA GTC CAC TCT GAT CCT-3′ antisense.

The locations of primers within the ALL1 and AF4 are reported elsewhere (23). Negative controls (reagents + water) were included in all PCR experiments.

Sequencing. The ALL1/AF4 PCR products were purified to remove the excess primers and dNTPs by electrophoresis in 1% agarose, and the DNA fragment was recovered by the Geneclean II purified method (BIO 101, La Jolla, CA). Purified DNA was then sequenced directly by the dideoxynucleotide chain termination method with a modified T7 DNA polymerase (Sequenase 2.0; United States Biochemical Corp., Cleveland, OH), using as sequencing primer either one of the two oligomers used for PCR amplifications. DNA (200–400 ng) and 1 pmol of primer were annealed in a 10-μl reaction by heating to 95°C for 7 min and cooling at room temperature for 3 min using 2 μl of 5 × sequencing buffer, 2 μl of 1:10 diluted dNTPs mix, 1 μl of 0.1 mol/liter DTT, 2 μl of 1:8 diluted Sequenase T7 DNA polymerase (from Sequenase 2.0 kit) and 0.5 μl [α-^{35}S]dATP (specific activity, >1500 Ci/mmol; Amersham, Buckinghamshire, United Kingdom). The termination reaction was performed by adding 3.5 μl of this extension mix to 2.5 μl of each termination mix (Sequenase 2.0 kit) and incubating for 3 min at 50°C. The reaction was stopped by the addition of 4 μl formamide dye to the mix. The samples were electrophoresed in denaturing sequencing gel containing 6% polyacrylamide and 7 mol/liter urea, fixed, dried, and exposed to X-ray film (XAR-5; Eastman Kodak, Rochester, NY) at room temperature for 3 days.

Results

Mapping of ALL1 Breakpoints in Infant, TR, and *de Novo* Leukemias. Seventy patients with ALL1-rearranged ALs were analyzed for breakpoint position within the bcr of the *ALL1* gene. Of these, 36 were infants, 29 were children and adults with *de novo* ALs, and 5 had TR ALs occurring after therapy that included topo II-inhibiting drugs for solid cancer (2 cases) or for Hodgkin's lymphoma (3 cases). The main clinical features of the 70 patients are reported in Table 1.

Using the B859 cDNA fragment as a probe, ALL1 rearrangements were detectable in all patients with at least one of the four restriction enzymes used. Abnormal or rearranged ALL1 restriction fragments were identified in 66 of 67 (98%) cases digested with *Bam*HI, 46 of 58 (79%) digested with *Bgl*II, 11 of 13 (85%) digested with *Hin*dIII, and 16 of 40 (40%) digested with *Xba*I.

As shown in Fig. 1*a*, two additional probes (B351 and FA4) were used to map the location of ALL1 breakpoints on Southern blot experiments. Due to the position of these two probes and the restriction map of the bcr, we were able to define the breakpoint location in the 46 cases showing *Bgl*II rearrangements. In fact, the two probes used in *Bgl*II-digested DNA facilitated the discrimination between 23

Table 2 *Clinical and biological features of the 30 patients studied for the ALL1/AF4 fusion transcript*

	Infants (<12 mo)	Others (3–65 yr)
Total cases	19	11
Sex		
Male	5	1
Female	14	10
Phenotype		
Early B[a]	17	10
Pre-B	2	1
WBC count		
≥100 × 10^9/liter	13	10
<100 × 10^9/liter	6	1
Hepatosplenomegaly		
Yes	14	9
No	5	2

[a] Early B, HLA-DR+/CD19+/CD10−; Pre-B, HLA-DR+/CD19+/CD10+.

cases with centromeric (B351 rearranged/FA4 germline) and 23 cases with telomeric ALL1 breakpoints (B351 germline/FA4 rearranged). We referred to these centromeric and telomeric ALL1 regions as region A (3.5 kb) and region B (4.8 kb), respectively (Fig. 1*a*). In 11 additional cases with a t(4;11) translocation but without informative Southern results or DNA available, it was nevertheless possible to identify ALL1 breakpoints by RT-PCR of the *ALL1/AF4* gene fusion cDNA followed by direct sequencing of the amplification products. By this strategy, 1 patient had ALL1 breakpoint in region A, and 10 had a breakpoint in region B. In summary, ALL1 breakpoint location was determined in 57 of 70 cases by Southern and/or RT-PCR of the ALL1/AF4 chimeric transcript.

The location of ALL1 breakpoints according to three major diagnostic subsets is illustrated in Fig. 1*b*. Eight of 28 (28%) infants, 16 of 24 (66%) *de novo*, and none of the 5 TR ALs had a breakpoint in region A, whereas 20 of 28 (71%) infant, 8 of 24 (33%) *de novo*, and 5 of 5 (100%) TR ALs had a breakpoint located in region B. The difference in breakpoint location among the three leukemic subsets was statistically significant ($P = 0.002$).

ALL1/AF4 Junction Types in Infant *versus* Noninfant Leukemias. The most common ALL1 fusion in infant leukemia is ALL1/AF4 with t(4;11)(q21;q23); almost all of these cases are diagnosed as acute lymphoblastic leukemia, whereas some have mixed lymphomyeloid lineage phenotype (24). ALL1/AF4 fusion transcript was detected by RT-PCR in 30 of 34 t(4;11)-positive ALs. Of these 30 cases, 19 were infants, and 11 were children or adults with *de novo* ALs. No RNA was available in the remaining cases. The main clinical and biological characteristics of these 30 patients are reported in Table 2.

Using our RT-PCR strategy, we detected variably sized amplification products of 286, 418, 491, and 532 bp, depending on breakpoint position and alternative splicing on both ALL1 and AF4 genes. Sequencing of the junction fragments showed that the 286-bp product resulted from fusion of ALL1 exon 6 to AF4 site A (*i.e.*, nucleotide number 1414 of AF4), the 418-bp product resulted from fusion of ALL1 exon 7 to AF4 site A, the 491-bp amplified fragment resulted from fusion of the ALL1 exon 8 to AF4 site A, and the 532-bp fragment resulted from fusion of ALL1 exon 8 to AF4 site B (*i.e.*, nucleotide number 1459 of AF4; Fig. 2). As illustrated in Fig. 3*b*, of the 11 noninfant cases, 9 had junctions between ALL1 exon 6 and AF4 site A, and 2 cases had junctions between ALL1 exon 7 and AF 4 site A. By contrast, of the 19 infants, 4 had the ALL1 exon 6-AF4 site A junction, 1 had the ALL1 exon 7-AF4 site A junction, and the remaining 14 had ALL1 exon 8/AF4 site A (12 cases) or ALL1 exon 8/AF4 site B (2 cases) junctions. This different breakpoint distribution between the two leukemic subsets was statistically significant ($P = 0.00005$).

On the basis of the RT-PCR results, which showed that all noninfant cases had ALL1 breakpoint in intron 6 or intron 7, we extended region A to the entire intron 7 (region A1). Conversely, region B was redefined as being limited to intron 8 because no infant cases had breakpoints outside this region (Fig. 3*a*).

Discussion

The characterization of DNA breakpoints involved in gene rearrangements is relevant to our understanding of human leukemogenesis. Several DNA motifs physiologically prone to recombination have been identified in the proximity of leukemia-associated gene breakpoints and may therefore provide genetic landmarks for sites of illegitimate recombination.

Strissel-Broeker *et al.* (13) reported a series of 39 patients with *ALL1* (*MLL*) gene rearrangements and fusions and were able to map the approximate intronic position of each breakpoint using Southern blotting with a combination of restriction enzymes and DNA probes. Their data indicated that whereas secondary ALs associated with prior

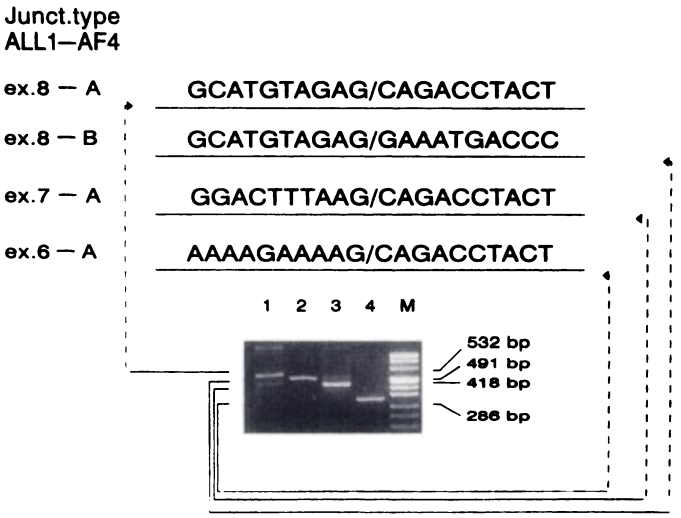

Fig. 2. Different ALL1/AF4 fusion types obtained by RT-PCR and direct sequence analysis of the junction segments. The size of variable junctions and nucleotide sequences are indicated for each type of amplified product.

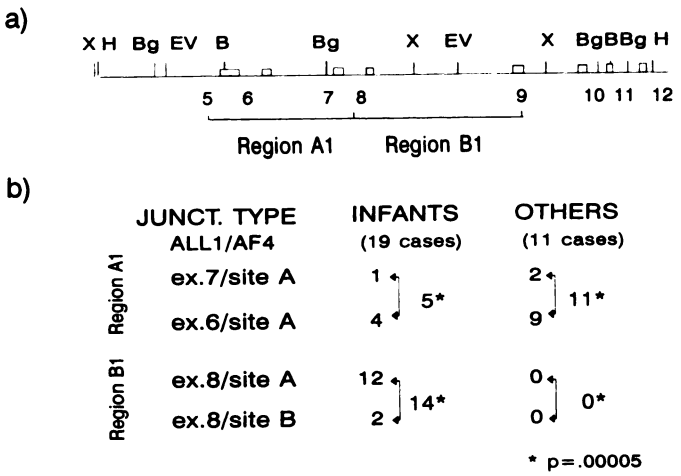

Fig. 3. *a*, new definition of the two major breakpoint regions into *regions A1* and *B1*. According to the RT-PCR data, the region originally defined as region A (Fig. 1) was extended to the entire intron 7 and named region A1, whereas region B (Fig. 1) was limited to intron 8 and renamed B1. *b*, the type of ALL1/AF4 junction as determined by RT-PCR in the three distinct patient categories.

exposure to topo II-inhibiting drugs had breakpoints biased toward the telomeric region (6 of 8 cases), patients with *de novo* ALs were more likely (23 of 31 cases) to have centromeric breaks. The breakpoint distributions were of considerable interest in relation to their proximity to motifs that might be involved in illegitimate recombination, particularly SARs and consensus sequences for topo II binding in exon 9, which might therefore provide the, or a, major site. Clearly, most breaks are distributed some distance away (hundreds of bp) from the major topo II site in exon 9, but the relative proximity of breaks in TR ALs to the region in which both the exon 9 site and other candidate topo II sites are concentrated suggests that this reflects the direct role of therapeutic drugs, such as the epidophyllotoxins and anthracyclines, in localized or preferential breaks in the ALL1 gene via the stabilization of the cleavable complex that topo II forms with replicated DNA (25). Moreover, the ability of topo II-inhibiting drugs to cleave double-stranded DNA at a site in exon 9 has been recently demonstrated *in vivo* and *in vitro* by Aplan *et al.* (26).

The corollary, then, is that most *de novo* ALs with ALL1 fusions, having more centromeric breaks, may arise through a mechanism that does not involve topo II. If correct, this interpretation would have obvious implications and, in particular, would favor recent suggestions that exposure of the fetus to natural or medicinal substances that interact with topo II during pregnancy might be a significant cause of *ALL1* gene rearrangements in infant AL (17, 18).

In a study by Strissel-Broeker *et al.* (13) only six of the *de novo* cases were infants with no clear bias of breakpoints, but the number of cases was probably to small to reveal whether or not bias existed in infant cases. The present study reports for the first time an analysis of the genomic ALL1 breakpoints in a substantial series of infant ALs compared with older patients with *de novo* and TR ALs. Our results confirm the finding of Strissel-Broeker *et al.* (13) insofar as all five patients with TR ALs had breakpoints clustering in the telomeric portion of the ALL1 bcr, whereas the majority (16 of 24) of patients with childhood or adult *de novo* ALs showed centromeric breakpoints. We used a *Bgl*II site to distinguish the two portions of the ALL1 bcr, instead of the 1.5 kb more telomeric *Xba*I site considered by Strissel-Broeker *et al.* (13). Interestingly, the centromeric portion of ALL1 bcr encompassed by *Bgl*II restriction sites contains the highest prevalence of Alu sequences and is therefore highly prone to recombinogenic events, as is also demonstrated by sequence analysis of several ALL1 breakpoints in patients with *de novo* ALs (15). Of the 11 *de novo* ALs here analyzed by RT-PCR of the ALL1/AF4 junction, 2 had an ALL1 breakpoint in intron 7 immediately telomeric to the *Bgl*II site and none had an ALL1 breakpoint in intron 8. On the basis of these findings, we considered it appropriate to extend the region of *de novo* ALL1 breakpoints to the entire intron 7 (Fig. 3*a*, *region A1*).

However, the key and novel observation in our study was the bias in *ALL1* gene breakpoints in infant cases, which is different from the bias in *de novo* cases that was reported previously (13). We found that breakpoints were localized in the telomeric part of the ALL1 bcr in the majority of cases (19 of 26). RT-PCR analysis of the ALL1/AF4 chimeric transcript performed in 15 of these 19 patients showed that 14 had breakpoints in intron 8.

These data and those published previously (13, 21, 27–29) indicate that although intronic breakpoints in the *ALL1* gene can be distributed over extensive intronic regions totaling around 10 kb, there is a significant bias in the distribution of sites between different types of ALs. However, the majority of cases of infant ALs, including lymphoblastic cases with t(4;11) or ALL1/AF4 fusions, have a similar telomeric bias in breakpoint position as secondary leukemias. These data lends further credence to the suggestion that exposures to topo II-inhibiting chemicals in pregnancy may be causally involved in leukemogenesis. A number of candidate agents have been listed (17–20, 30) and this possibility is being pursed in case-control epidemiological studies. Prior epidemiological evidence has identified significant associations between a number of pregnancy events and subsequent infant leukemia, including maternal intake of marijuana or alcohol, exposure to pesticides, or prior fetal loss (17–20). Although the cases involved in these studies were not classified with respect to *ALL1* gene status, it is possible that the excess of pregnancy exposures or events observed reflected an impact of topo II inhibitors (18–20). More recently, Ross *et al.* (17) have reported another case-control study that revealed a significant association between high dietary intake of natural topo II inhibitors (flavonoids) and risk of infant leukemia.

Our own studies in this context have taken the form of an international collaborative case-control study of pregnancy events in which all cases are classified with respect to *ALL1* gene involvement (30). These epidemiological studies, in concert with further biological investigations, raise the prospect of identifying a preventable causal mechanism in one of the types of leukemia that is clinically most intractable: infant acute lymphoblastic leukemia.

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