Molecular emergence of acute myeloid leukemia during treatment for acute lymphoblastic leukemia

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Therapy-related acute myeloid leukemias (t-AML) with translocations of the *MLL* gene are associated with the use of topoisomerase II inhibitors. We established the emergence of the malignant clone in a child who developed t-AML with a t(11;19) (q23;p13.3) during treatment for acute lymphoblastic leukemia (ALL). The *MLL-ENL* and the reciprocal *ENL-MLL* genomic fusions and their chimeric transcripts were characterized from samples collected at the time of t-AML diagnosis. We used PCR with patient-specific genomic primers to establish the emergence of the *MLL-ENL* fusion in serially obtained DNA samples. The *MLL-ENL* fusion was not detectable in bone marrow at the time of ALL diagnosis or after 2 months of chemotherapy (frequency $<8.3 \times 10^{-7}$ cells^{-1}). The genomic fusion was first detected in bone marrow after 6 months of treatment at a frequency of one in 4,000 mononuclear bone marrow cells; the frequency was one in 70 cells after 20 months of therapy. At the first detection of *MLL-ENL*, the only topoisomerase II inhibitors the patient had received were one dose of daunorubicin and two doses of etoposide. The *MLL-ENL* fusion was not detectable in blood at the time of ALL diagnosis or after 0.7, 2, 8, 10, and 12 months of therapy but was detectable in blood at 16 months (one in 2.3×10^4 cells). Recombinogenic *Alu* sequences bracketed the breakpoints in both fusions. These data indicate that the malignant clone was not present before therapy, arose early during chemotherapy, and was able to proliferate even during exposure to antileukemic therapy.

Therapy-related acute myeloid leukemia (t-AML) is a devastating complication of antineoplastic chemotherapy. There are two recognized categories of therapy-related leukemia: one associated with alkylating agents and the other associated with topoisomerase II inhibitors (1). Topoisomerase II inhibitors are widely used to treat leukemias, lymphomas, testicular, breast, and ovarian cancers. t-AML associated with topoisomerase II inhibitors has a short latency [median 24–34 months (2, 3), as short as 10 months (4)], and a predominance of myelomonoblastic and monocytic morphologic features. Despite the epidemiological link, the roles of schedule and dose for topoisomerase II inhibitors in the pathogenesis of t-AML remain unclear.

Interestingly, over 70% of the cases of t-AML that are associated with exposure to topoisomerase II inhibitors have translocations involving the *MLL* gene on chromosome 11-band q23 (5, 6), resulting in the in-frame fusion of *MLL* with any one of more than 40 partner genes (7). The resulting *MLL* chimeric products may act through a gain-of-function mechanism and are important for leukemic transformation (8); haploinsufficiency for wild-type *MLL* allele may also play a role during malignant transformation (9). t-AML occurs in up to 10% of children treated for acute lymphoblastic leukemia (ALL) (1, 2, 10), thereby constituting a significant proportion of treatment failures.

Therapy of childhood ALL is unique in that it comprises hundreds of doses of chronically administered chemotherapy, given weekly or daily, for 2–3 years. The use of topoisomerase II inhibitors to treat ALL is controversial, particularly the leukemogenic epipodophyllotoxins etoposide and teniposide, especially in view of improved event-free survival (≈80%) in some contemporary clinical trials (11). The incorporation of the anthracycline-type topoisomerase II inhibitors into ALL remission induction and reinduction regimens, a common approach (11), is also controversial because they have also been implicated in t-AML (12–14). t-AML has not been clearly linked to cumulative doses of topoisomerase II inhibitors (15, 16), having been reported after regimens that contain only a few doses of the agents (14, 17). Thus, the timing of the induction of *MLL* rearrangements, relative to the few doses of topoisomerase II inhibitors that may be used for lower-risk ALL, is of considerable importance in devising ALL treatment regimens.

Herein, we report the molecular emergence of t-AML during treatment for ALL. We sequenced the *MLL-ENL* fusion and the reciprocal *ENL-MLL* from DNA samples at the time of t-AML diagnosis. Using patient-specific primers, we backtracked the emerging malignant clone in bone marrow and peripheral blood serially collected during ALL therapy. Our data document that there was no evidence of AML before starting ALL therapy (frequency $<8.3 \times 10^{-7}$ cells^{-1}) and demonstrate the early development of the *MLL-ENL* fusion, occurring after only three low doses of topoisomerase II inhibitors.

Case. A 16-year-old boy with B-lineage ALL presented with 98% lymphoblasts in bone marrow and a leukemic cell DNA index of 1.22. He had no detectable blasts in cerebrospinal fluid and no signs of testicular involvement. The bone marrow karyotype at diagnosis of ALL was hyperdiploid: 58, XY,+X,+X,+4,+8,+10,+12,+17,+18,+20,+21,+21,+22. Immunophenotyping was as described (18). The presence of extra chromosomes was confirmed by fluorescence *in situ* hybridization (Chromoprobe Interphase, Rainbow Scientific, Windsor, CT). The bone marrow was negative for the following translocations and their fusion transcripts: t(9;22) [p210 *BCR-ABL* and p190 *BCR-ABL*], t(1;19) *E2A-PBX1*, t(4;11) *MLL-AF4*, and t(12;21) *TEL-AML1* (19).

The patient was enrolled on the Total Therapy Study XIII B at St. Jude Children's Research Hospital. Informed consent was obtained from the patient and his guardian. He was assigned to the low-risk treatment arm and received window therapy with methotrexate (1 g/m^2 i.v.) for 24 h and 6-mercaptopurine (1 g/m^2 i.v.) over 6 h. He received 6 weeks of remission induction

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Abbreviations: t-AML, therapy-related acute myeloid leukemia; ALL, acute lymphoblastic leukemia; G-CSF, granulocyte colony-stimulating factor; TPMT, thiopurine methyltransferase; NQO1, NAD(P)H/quinone oxidoreductase.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. AF373585, AF373586, AF373587, and AY040555).

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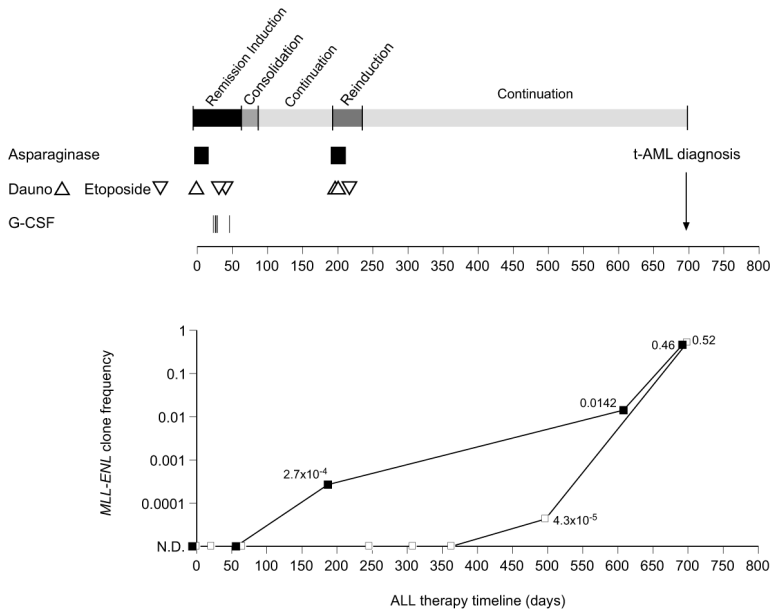

Fig. 1. Treatment protocol (St. Jude Children's Research Hospital Total XIII B) for ALL in a patient who developed t-AML (*Upper*). The time of t-AML diagnosis is indicated by an arrow. The interval corresponding to each therapy block is indicated in gray scale boxes (*Upper*) and corresponds with the time line in days (*Lower*). The times of administration of topoisomerase II inhibitors (etoposide, 300 mg/m^2 ▽, and daunorubicin, 25 mg/m^2 Δ), asparaginase, 10,000 units/m^2 thrice weekly × six doses (■), and G-CSF (|) are indicated. *MLL-ENL* genomic clone frequencies (*Lower*) for bone marrow (■) and for peripheral blood samples (□). N.D., not detected.

therapy with prednisone 40 mg/m^2 daily, daunorubicin 25 mg/m^2 on day 1, vincristine 1.5 mg/m^2 i.v. weekly, asparaginase 10,000 units/m^2 i.m. three times per week × six doses, and etoposide 300 mg/m^2 plus cytarabine 300 mg/m^2 on days 35 and 41 (Fig. 1). Because of a preexisting infection and severe mucositis and neutropenia, the patient did not receive two other scheduled doses of topoisomerase II inhibitors, and he received five doses of granulocyte colony-stimulating factor (G-CSF) 5 μg/kg during remission induction. On attaining clinical remission, he received weekly methotrexate 2 g/m^2 i.v. × 2 and daily oral mercaptopurine 75 mg/m^2. Continuation therapy consisted of weekly blocks of chemotherapy: daily oral mercaptopurine 75 mg/m^2 and weekly methotrexate 40 mg/m^2, i.v. or i.m.; daily oral dexamethasone 8 mg/m^2/day plus vincristine 1.5 mg/m^2 every 4 weeks; and methotrexate 2 g/m^2 i.v. and daily oral mercaptopurine 75 mg/m^2 every 8 weeks during the first year. Reinduction therapy, given from weeks 28 to 34, was the same as induction therapy, except that two doses of daunorubicin (days 1 and 8) and only one dose of etoposide plus cytarabine (day 22) were given. The patient received 14 doses of intrathecal chemotherapy during the first year.

Twenty-three months into therapy, he was diagnosed with t-AML (FAB M4), confirmed by extensive immunophenotyping (18). The bone marrow karyotype at the time of diagnosis of t-AML was 46,XY,t(11;19)(q23;p13.3) in 60% of the metaphase cells; blasts were positive for *MLL-ENL* fusion transcripts by reverse transcription–PCR (20) and negative for *MLL-AF4*, *MLL-AF9* and *MLL-ELL* transcripts.

He received an allogeneic stem cell transplant and died of complications (graft-versus-host disease) 7 months later.

Pharmacokinetics and Pharmacogenetics. The patient was genotyped for the presence of mutations *TPMT *2, *3A, *3B*, and **3C* in thiopurine methyltransferase (TPMT) (21). TPMT activity was measured in red blood cells (22). Blood DNA was also genotyped for a polymorphism in the *CYP3A4* promoter (23) and in NAD(P)H/quinone oxidoreductase (NQO1) (24). DNA from bone marrow at the time of diagnosis of t-AML was analyzed for point mutations in the cytoplasmic domain of the G-CSF receptor (25).

Plasma clearance of etoposide and area under the plasma concentration vs. time curve of etoposide and its catechol metabolite were measured during reinduction therapy, as described (26).

Samples. DNA and RNA were isolated by personnel not involved in further PCR analysis in a laboratory free of PCR amplification products.

Blood and bone marrow mononuclear cells were collected by centrifugation on a density gradient (Lymphoprep, Nycomed, Oslo). DNA and RNA were isolated by using TriReagent (Molecular Research Center, Cincinnati). DNA was dissolved in Tris-EDTA buffer and stored at 4°C until use. RNA was dissolved in water and stored at −70°C until use.

Cloning *MLL-ENL* and *ENL-MLL* Breakpoints. Long-distance inverse PCR (27) was used to amplify the *MLL-ENL* breakpoint from 100 ng of DNA from blood (43% blasts) at the time of diagnosis of t-AML, by using the Expand Long Template PCR system (Roche Molecular Biochemicals). *MLL* "inverse" primers were: primer A, 5′-ATACATCCCTGAGAAATGGCAGAGA-AAC-3′ (position 708–681, GenBank no. U04737) and primer B, 5′-AGCACCAACTGGGGGAATGAATAAGAAC-3′ (position 2576–2603) (Fig. 2). Conditions (MJ Research Thermal Cycler, Watertown, MA) were: 95°C × 5 min; 45 cycles at 95°C × 30 s, 58°C × 1 min, and 68°C × 12 min; extension at 70°C × 10 min. The PCR products spanning the *MLL-ENL* breakpoint were sequenced (ABIprism 3700, Applied Biosystems) and analyzed with GCG Ver. 10.1. Sequence motifs (e.g., *Alu* repeats, topoisomerase II sites) were analyzed [e.g., FINDPATTERNS (www.gcg.com), CENSOR (http://www.girinst.org/Censor_Server.html), MAR FINDER (http://www.futuresoft.org/MAR-Wiz/)]. The reciprocal *ENL-MLL* breakpoint was amplified by using 100 ng of DNA from bone marrow at the time of t-AML, by using the Expand Long Template PCR system (Roche Molecular Biochemicals). Patient-specific primers to cover the putative breakpoint were: 5′-ATCAGCCCACTA-CAACCTCCAC-3′ (forward) and 5′-AAAACAGACAC-CCTCCCTTCAC-3′ (reverse). Touchdown PCR conditions were 92°C × 2 min, 5 cycles of 92°C × 15 s, 65°C (−1°C per cycle) × 35 s and 68°C × 3 min; then 30 cycles of 92°C × 15 s, 60°C × 30 s and extension at 68°C. PCR products were analyzed as for the *MLL-ENL* genomic fusion.

***MLL-ENL* and *ENL-MLL* Fusion Transcripts.** One microgram of RNA was reverse transcribed with Superscript (GIBCO/BRL) by using oligo(dT)$_{12–18}$ primers.

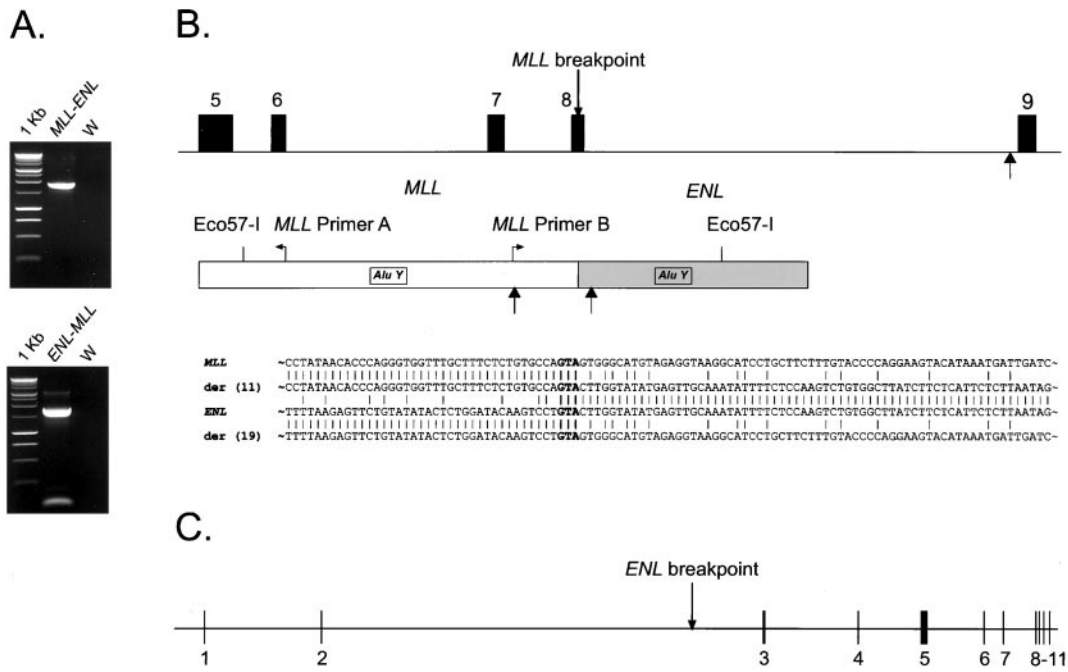

Fig. 2. (*A*) Long-distance inverse PCR amplification of a 1.9-kb product containing the *MLL-ENL* fusion from peripheral blood DNA at the time of diagnosis of t-AML (*Upper*) and of a 1.8-kb product containing the *ENL-MLL* fusion from bone marrow at the time of t-AML diagnosis (*Lower*). W, water control. DNA marker: 1-kb ladder. (*B*) Schematic of *MLL* (*Top*), indicating exons 5–9, the location of the *MLL* breakpoint in this case, and the location of an *in vivo* topoisomerase II cleavage site (46) by an arrow. The *MLL-ENL* breakpoint region amplified by long-distance inverse PCR (*Middle*) is indicated. The positions of the *MLL* inverse primers (*A* and *B*), the Eco57-I restriction sites, the *Alu Y* repeats, and two *in vitro* topoisomerase II recognition sites (black arrows located below the fusion product) are indicated. Details of the *MLL*, der (11), *ENL*, and der (19) DNA sequences bracketing the fusion points are depicted (*Bottom*). The triplet GTA in bold depicts the fusion points in both parental alleles and in the fusions. (*C*) Genomic organization of *ENL*, covering ≈58 kb, derived from alignment of genomic and cDNA sequences (GenBank no. L04285). Boxes show 11 exons. The *ENL* breakpoint corresponds to position 46,181 (GenBank no. NT_011316).

For amplification of the *MLL-ENL* transcripts, primers were: forward 5′-CAATAAGCAGGAGAATGCAGG-3′ (position 2370–2390, GenBank no. U04737) and reverse 5′-GGAATTGTGGGTAACATGGGG-3′ (position 494–474, GenBank no. L04285). For amplification of the *ENL-MLL* transcripts, primers were: forward 5′-GTTAGAGCTGGGGCATCGC-3′ (position 30–48, GenBank no. L04285) and reverse 5′-TTGTGGGTTTGGTGGGGTAG-3′ (position 8045–8026, GenBank no. U04737). Each 50-μl PCR contained 2 μl of the cDNA conversion mixture, nuclease-free water, 50 pmol of each primer, 2.5 units of Amplitaq Gold DNA polymerase (Perkin–Elmer), 250 μM each dNTP (Invitrogen), and Gene Amp PCR buffer (Perkin–Elmer). Amplification conditions were: 95°C × 1 min; 45 cycles of 95°C × 30 s, 53°C × 30 s, and 70°C × 45 s; and final extension at 70°C × 10 min. Reverse transcription–PCR products were sequenced by using the PCR primers.

PCR Analysis in Serially Collected Samples. Reagents, equipment, and samples were isolated from potential sources of contamination by use of separate laboratories and Clean Spot PCR work stations (Coy Laboratory, Grass Lake, MI). Nested PCR was used to amplify *MLL-ENL* in DNA samples collected during ALL therapy. First-round primers were: 5′-AGCAGCAGTTATTTTTGGACTC-3′ (forward) and 5′-GCCTCCCTTACTAGATACCCAC-3′ (reverse); second round primers were: 5′-GAAAATGTGTGGGAGATGGGAG-3′ (forward) and 5′-CAAGTGTGGCAAAGGGTTTCAG-3′ (reverse). Each first-round 50-μl PCR reaction contained 100 ng of DNA, nuclease-free water, 50 pmol of each primer, 1.25 units of Amplitaq Gold, 250 μM each dNTP, and Gene Amp PCR buffer. The frequency of the *MLL-ENL* rearrangement can be estimated by using the principles of limiting dilution and Poisson statistics (28). Assuming 100 ng of DNA represents 16,000 cells, a single PCR with 100 ng of DNA comprising a 10^4 dilution of *MLL-ENL*-carrying cells in *MLL-ENL*-negative cells would have 80% of replicate PCRs positive, and a 10^5 dilution would have only 15% positive (28). These frequencies agree with those observed (data not shown), substantiating the assumption that each PCR amplified *MLL-ENL* if it was present. Amplification conditions were 95°C × 5 min; 45 cycles of 95°C × 45 s, 53°C × 1 min, 70°C × 1 min; and a final extension at 70°C × 10 min. One microliter of the first-round PCR was reamplified by using identical conditions with the nested primers. The integrity of each DNA sample for PCR was confirmed by using internal *MLL* primers: forward 5′-AGCACCAACTGGGGGAATGAATAAGAAC-3′ (position 2576–2603, GenBank no. U04737) and reverse 5′-CTCAGACACGGACTATTAAAAGGCTCAC-3′ (position 2875–2848), with the same PCR conditions as for *MLL-ENL*.

Results

Sequencing of long-distance inverse PCR products at diagnosis of t-AML (Fig. 2) revealed that the breakpoint occurred at position 3131 of the *MLL* breakpoint cluster region (GenBank no. U04737). The breakpoint is in exon 8, 14 bases upstream from its 3′ end (Fig. 2). The 3′ portion of the breakpoint mapped to chromosome 19 (GenBank no. NT_011316), with the breakpoint at position 46,181. To obtain some insight about the genomic organization and the location of the breakpoint, we compared the cDNA sequence of *ENL* (GenBank no. L04285) and the partial genomic sequence of chromosome 19. *ENL* exon positions were confirmed and refined by the use of National Center for Biotechnology Information ACE VIEW (Fig. 2). The analysis indicated that the *ENL* gene contains 11 exons and that the breakpoint is located in intron 3, which contains 31,512 bp and is the largest intron in *ENL* (Fig. 2).

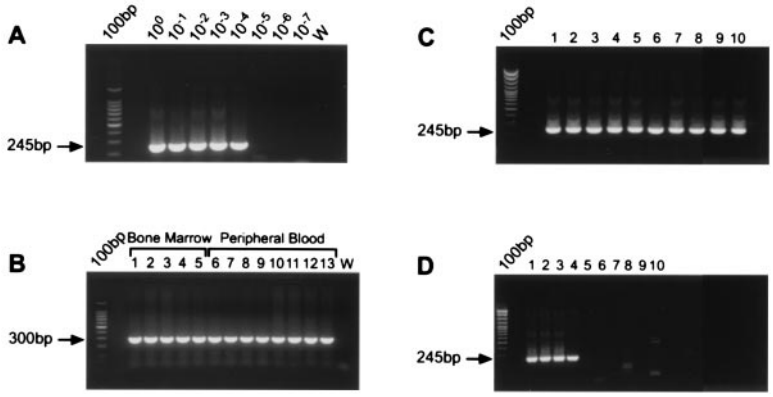

Fig. 3. (*A*) Limit of detection of the *MLL-ENL* genomic PCR reaction. Second-round PCR amplification of a 245-bp *MLL-ENL* breakpoint from t-AML diagnostic blood DNA (100 ng–0.01 pg) serially diluted in normal blood DNA. W, water. 100 bp = DNA ladder. (*B*) DNA integrity control for samples serially collected during ALL therapy. PCR amplification of a 300-bp segment of *MLL* gene. Lanes 1–5, bone marrow DNA; lanes 6–13, blood DNA. W, water. 100 bp = DNA ladder. (*C*) Second-round PCR of a 245-bp *MLL-ENL* breakpoint from bone marrow DNA at 608 days of ALL therapy. All of the replicates (100 ng each) were positive and were run with positive and negative controls (see details in the text). (*D*) Second-round PCR of a 245-bp *MLL-ENL* breakpoint from blood DNA collected after 497 days of ALL therapy. Four of 10 replicates (100 ng each) were positive and were run with positive and negative controls.

The *MLL-ENL* breakpoint is flanked by two *Alu Y* repeats (29) (Fig. 2). One *Alu Y* repeat (290-bp long, DNA-positive strand) is in *MLL*, 1,422 bp 5′ from the breakpoint. An *Alu Y* repeat (290-bp long, DNA-negative strand) on the *ENL* side is located 630 bp 3′ of the *MLL-ENL* breakpoint. Both *Alu Y* segments contain the core sequence 5′-CCTGTAATC**CCAG-**CACTTTGGGAGGC-3′ (30); one G→A transition (position 25) was found in the core element located in *ENL*. The breakpoint is flanked by two topoisomerase II sites 5′-A/GNT/CNNCNNGT/CNGG/TTNT/CNT/C-3′ (31); the site in *MLL* (9/10 matches, DNA-positive strand) is located 523 bp from the breakpoint, and the site in *ENL* (9/10 matches, DNA-positive strand) is 101 bp 3′ of the breakpoint (Fig. 2).

The derivative *ENL-MLL* genomic fusion was amplified from bone marrow DNA at the time of t-AML diagnosis (Fig. 2). Interestingly, the *ENL-MLL* breakpoint is also flanked by two *Alu Y* repeats containing the core element. One *Alu Y* repeat is in *ENL* (288 bp long, DNA-negative strand) 758 bp 5′ from the breakpoint; the other (290 bp long, DNA-positive strand) is in *MLL* 842 bp 3′ from the fusion point. Three topoisomerase II sites (9/10 matches, DNA-positive strand) were found in *ENL* at a distance of 56, 153, and 351 bp 5′ from the fusion point, respectively.

The triplet GTA is present on both *MLL-ENL* and *ENL-MLL* genomic fusion points and on the sequences of both parental genes. Both fusions indicate that the fusion event occurred in a conservative manner without gain or loss of DNA from either *MLL* or *ENL* (Fig. 2).

The *MLL-ENL* transcript has an in-frame fusion of *MLL* exon 7 to *ENL* exon 3. The *ENL-MLL* transcript contains *ENL* exon 2 fused in frame to *MLL* exon 9. No *MLL* exon 8 was found in either the *MLL-ENL* or *ENL-MLL* transcripts, indicating that the genomic translocation prevented exon 8 expression.

The sensitivity of the PCR reaction to track the emergence of the *MLL-ENL* fusion during therapy was tested by diluting the t-AML diagnostic blood DNA in normal leukocyte DNA (100 ng). The PCR reaction was sensitive enough to detect one cell carrying the *MLL-ENL* fusion per PCR reaction, which was tested against a background of 16,000 cells without the fusion (Fig. 3*A*). Specificity was demonstrated by the absence of *MLL-ENL* fusions in DNA samples from five normal volunteers (data not shown).

DNA samples from bone marrow ($n = 5$) and blood ($n = 8$) collected during ALL therapy were analyzed for the *MLL-ENL* fusion (Fig. 3 *C* and *D*). Each experiment included at least as many PCR negative controls (water) as it did PCRs with patient DNA, and negative controls of 100 ng of DNA from normal volunteers were included in each experiment. We tested 10 replicates for each of the blood DNA samples at days 21, 65, and 246 of ALL therapy; for the remainder of the blood and bone marrow samples, the average number of replicates tested per sample was 40, $\approx 6.4 \times 10^5$ cells per sample.

The *MLL-ENL* genomic fusion was not detectable in bone marrow at diagnosis of ALL or in bone marrow 56 days after the start of ALL therapy. As each PCR reaction could detect the *MLL-ENL* fusion if it was present (see *Methods*, PCR Analysis in serially collected samples), by increasing the number of replicates (75 × of 100 ng at both times), we confirmed the absence of the *MLL-ENL* fusion in an amount of DNA corresponding to $\approx 1.2 \times 10^6$ bone marrow cells for each of the early samples, indicating that the frequency was likely to be less than 8.3×10^{-7} cells^{-1} at diagnosis and at the end of remission induction.

The *MLL-ENL* genomic fusion was detected in bone marrow after 6 months (day 187) of chemotherapy, just before remission reinduction. The frequency of the *MLL-ENL* clone was estimated by limiting dilution (28) at 2.7×10^{-4} cells^{-1} (1 in 4,000 cells) (Fig. 1). The fusion was also detectable in bone marrow after 20 months (day 608, Fig. 3*C*) at a frequency of 0.0142 cells^{-1} (1 in 70 cells) and at the time of t-AML diagnosis at 0.46 cells^{-1}.

The *MLL-ENL* fusion was not detected in DNA from blood at the time of diagnosis of ALL or after 0.7, 2, 8, 10, and 12 months of ALL therapy (Fig. 1). The *MLL-ENL* fusion was detected in a blood sample after 16 months (day 497, Fig. 3*D*) at 4.3×10^{-5} cells^{-1} (1 in 2.3×10^4). The clone frequency at the time of diagnosis of t-AML in peripheral blood was 0.52, consistent with the 43% of blast cells established by morphology.

The *CYP3A4* promoter genotype was wild type; inactivating mutations in *TPMT* (nucleotides 238, 460, and 719) were not present; and there were no point mutations in the cytoplasmic domain of the G-CSF receptor. The genotype for *NQO1* (a C→T change at nucleotide 609) was homozygous variant. The clearance for etoposide was 2.2 l/h/m^2, and the areas under the plasma concentration vs. time curve for etoposide and its catechol metabolite were 229 μM·h and 1.88 μM·h, respectively, comparable to those of other patients treated at the same time point in therapy.

Discussion

The present study demonstrates the emergence of a t-AML-specific *MLL-ENL* genomic fusion after only three low doses of topoisomerase II inhibitors in a child with low-risk ALL. This represents, to our knowledge, the first temporal characterization and quantitative evaluation of the emergence of a t-AML-related fusion during ALL therapy. The early onset of a t-AML-related fusion was also demonstrated in a child with neuroblastoma (14).

The use of the *MLL-ENL* gene fusion as a specific leukemia-associated marker is supported by evidence showing that the resulting MLL-ENL chimeric proteins are etiologically impor-

tant in leukemias (32). The reciprocal *ENL-MLL* fusion occurred with no loss or gain of genetic material relative to the *MLL-ENL* fusion, and the reciprocal *ENL-MLL* transcripts were also present. The biological relevance of these reciprocal fusion transcripts remains to be elucidated.

We studied the timing of the fusion in DNA samples spanning 700 days of therapy for ALL. The *MLL-ENL* genomic fusion was not detected in either bone marrow or blood at the time of diagnosis of ALL nor was it detected at the end of remission–induction therapy. The final "doubling time" of the *MLL-ENL* bearing clone was 18.3 and 15.0 days in the bone marrow and peripheral blood, respectively, in the few months preceding diagnosis of t-AML, whereas the growth appeared to be slower (doubling time: 73.6 days) between days 187 and 608 of therapy. It is conceivable that the intense chemotherapy given during reinduction (starting at day 199) had an antiproliferative effect on the *MLL-ENL* clone. The increase in the *MLL-ENL* clone frequency during continuation chemotherapy indicates that the regimen was ineffective to stop the growth of the malignant clone. Whether the earlier detection in bone marrow relative to blood is because of the predilection of myeloid leukemia to localize in the bone marrow or because of a chemotherapy-induced suppression of migration of the myeloid cells from bone marrow to the periphery cannot be ascertained.

There are at least two theories to explain the early onset of the *MLL-ENL* clone in the context of this ALL regimen. One is that the topoisomerase II inhibitors directly caused the translocation event. That 70% of t-AML cases associated with the use of topoisomerase II inhibitors have *MLL* translocations compared with almost no cases of "alkylator-associated" AML supports the notion that the topoisomerase II inhibitors cause the rearrangements. However, we cannot rule out the possibility that a cell carrying the translocation was already present at the time of ALL diagnosis at a frequency of less than one in 1.2×10^6 cells, and that the clone was "selected for" by the chemotherapy. In another case of early-onset of t-AML (14), the translocation was not detected in pretherapy bone marrow slides, although the number of cells tested was far fewer than the numbers we were able to test. That many effective antileukemic regimens that do not contain topoisomerase II inhibitors are not able to "select for" such *MLL* rearrangements, and that the t-AML remains responsive to topoisomerase II inhibitors, argue against the theory of clonal selection (3).

Other risk factors such as concurrent chemotherapy and host-related factors might contribute to the development of t-AML (1, 33). For example, the use of asparaginase has been linked to an increased risk for t-AML (34, 35), and this patient had asparaginase concomitantly with or before the topoisomerase II inhibitors (Fig. 1).

Pharmacogenetic polymorphisms can account for interindividual variability in response to anticancer drugs (36), which may contribute to the risk of t-AML. A paucity of polymorphisms in the *CYP3A4* promoter was associated with the risk of t-AML (23), and in accord with this finding, the patient had a wild-type *CYP3A4* promoter genotype. A polymorphism at position 609 of *NQO1* has been associated with an increased risk of leukemias with *MLL* fusions (37) and also with therapy-related myeloid leukemia (24). The patient had a genotype that dictates the absence of NQO1 protein and enzymatic activity (38). Thus, the patient may have been particularly susceptible to the mutagenic effects of the chemotherapeutic agents.

The patient's pharmacokinetic parameters (clearance and area under the plasma concentration vs. time curve) for etoposide and its catechol were close to the median for this population, in agreement with the lack of differences in etoposide disposition between patients who did and did not develop t-AML (26). Patients with etoposide-related AML tend to have low TPMT activity (26, 39), but this patient had wild-type TPMT activity and genotype.

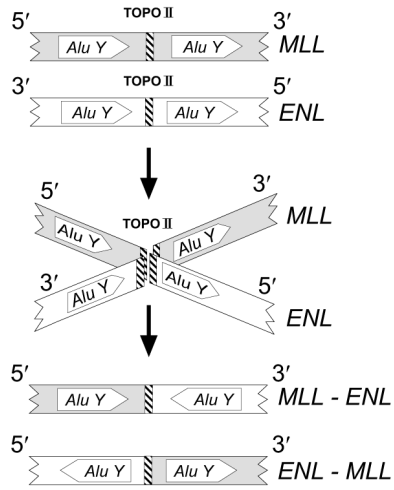

Fig. 4. Model for the genesis of the *MLL-ENL* and *ENL-MLL* fusions during ALL treatment with topoisomerase II inhibitors. Both *MLL* and *ENL* gene fragments originate by DNA double-strand breaks and are positioned by *Alu Y* repeats (*Top*). Double-strand DNA cleavage in *MLL* and *ENL* (*Middle*) is catalyzed by topoisomerase II (TOPO II) and stabilized by topoisomerase II inhibitors. Recombination processes resolve the breaks (*Bottom*), with *MLL-ENL* and *ENL-MLL* partners aligned regionally by *Alu Y* elements and locally by a region of short homology (the GTA triplet in the parental alleles and in the fusions is indicated by a crosshatched vertical bar). The resulting derivative fusions contribute to leukemogenesis and are present at the time of t-AML diagnosis.

Point mutations in the G-CSF receptor gene have been reported in patients who developed myeloid leukemia after chronic use of G-CSF (40). Because this patient received therapy with G-CSF during remission induction (Fig. 1), we sequenced this region but found no point mutations in the germline or in t-AML blast DNA. The contribution of G-CSF to the risk of t-AML is controversial (41).

Chromosomal breakage results from the stabilization of DNA/topoisomerase II complexes by topoisomerase II inhibitors (42). The sequences of the *MLL-ENL* and *ENL-MLL* breakpoints indicate that the fusion process occurred in a conservative manner, with a total preservation of the sequence corresponding to each one of the partners. Both *MLL-ENL* and *ENL-MLL* breakpoints are flanked by *Alu Y* repeats that are symmetrically positioned relative to each fusion point (Fig. 4). The *Alu Y* repeats on *ENL* are in an antiparallel orientation with respect to the *Alu Y* repeats of *MLL*. The *Alu* repeat elements contain the *Alu* core associated with recombination. The pentanucleotide motif CCAGC, which was contained in all four core elements, may stimulate the recombination processes (30). *Alu* repetitive elements have been found in other *MLL* gene rearrangements (14, 43–45). Whether topoisomerase II recognition sites near the breakpoints in fusions contributed to their genesis is not clear, as none of the sites precisely correspond to the breakpoints, and the function of such *in vitro* sequences has been questioned. An *in vivo* topoisomerase II cleavage site (46) is depicted in Fig. 2, and although it is well 3′ of the breakpoint, such cleavage has been hypothesized to participate in the genesis of *MLL* fusions. Assuming that cleavage occurred at the fusion breakpoint in both genes, the antiparallel *Alu Y* repetitive elements could have facilitated the alignment of *MLL* and *ENL*. The resulting reciprocal fusion may have occurred as an attempt to resolve the DNA cleavage caused by drug action (Fig. 4). That there are no insertions or deletions in either the *MLL-ENL* or *ENL-MLL* fusions indicates that both DNA breaks must have been remarkably stabilized, and that cellular recombination machinery in joining the nonhomologous partners was relatively

uncomplicated, without the "nibbling" or insertions associated with other translocations. The GTA triplet present at the *MLL* and *ENL* breakpoints suggests the ultimate role of DNA end-joining, as has been proposed for other *MLL* translocations (14, 47).

Therapy-related malignancies are devastating complications that account for a significant proportion of ALL treatment failures in children. The evidence presented here indicates that the malignant clone was present in bone marrow early during ALL chemotherapy. We hypothesize that the use of topoisomerase II inhibitors triggered the fusion event, a necessary step for malignant transformation. Our data are consistent with the notion that modest exposure to topoisomerase II inhibitors, in a permissive setting, can be sufficient to induce t-AML.

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