Chromosome translocations and covert leukemic clones are generated during normal fetal development

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Studies on monozygotic twins with concordant leukemia and retrospective scrutiny of neonatal blood spots of patients with leukemia indicate that chromosomal translocations characteristic of pediatric leukemia often arise prenatally, probably as initiating events. The modest concordance rate for leukemia in identical twins (≈5%), protracted latency, and transgenic modeling all suggest that additional postnatal exposure and/or genetic events are required for clinically overt leukemia development. This notion leads to the prediction that chromosome translocations, functional fusion genes, and preleukemic clones should be present in the blood of healthy newborns at a rate that is significantly greater than the cumulative risk of the corresponding leukemia. Using parallel reverse transcriptase–PCR and real-time PCR (Taqman) screening, we find that the common leukemia fusion genes, *TEL-AML1* or *AML1-ETO*, are present in cord bloods at a frequency that is 100-fold greater than the risk of the corresponding leukemia. Single-cell analysis by cell enrichment and immunophenotype/fluorescence *in situ* hybridization multicolor staining confirmed the presence of translocations in restricted cell types corresponding to the B lymphoid or myeloid lineage of the leukemias that normally harbor these fusion genes. The frequency of positive cells (10^{-4} to 10^{-3}) indicates substantial clonal expansion of a progenitor population. These data have significant implications for the pathogenesis, natural history, and etiology of childhood leukemia.

Chimeric fusion genes generated by chromosomal translocations are consistent genetic abnormalities in pediatric acute leukemia (1–3). DNA breaks and fusions occur in introns, and each patient's leukemic cells have a unique or clonotypic breakpoint, providing a specific, sensitive, and stable marker for tracking leukemic clones (4). Using this approach in identical twins with concordant leukemia (5–7) and, retrospectively, with neonatal blood spots (8–10), it was demonstrated that common fusion genes—*MLL* fusions in infants with acute lymphoblastic leukemia (ALL) and *TEL-AML1* and *AML1-ETO* in children with ALL or acute myeloblastic leukaemia (AML), respectively—arise predominantly *in utero*, probably as initiating events, and are present before and at birth in circulating blood.

The evolutionary, clonal development of pediatric cancers involves a sequence of two or more independent genetic events (11), and it therefore is unlikely that fusion genes, initiating the disease, would be sufficient. This supposition is supported by the modest concordance rate for ALL in monozygotic twin children, 5–10% (12), by protracted postnatal latency (up to 14 years) (7) and by the absence of overt signs of leukemia in mice transgenic for *AML1-ETO* (13) or *TEL-AML1* (14). This finding leads to the prediction that chromosomal translocations, functional fusion genes, and preleukemic clones should be generated in stem cells during fetal hemopoiesis, and present in blood at birth, at a rate that substantially exceeds the known incidence rate or cumulative risk of ALL or AML. The latter figures are ≈1 in 2,000 for any type of acute leukemia (0–15 years); ≈1 in 10,000 for ALL with *TEL-AML1*, and ≈1 in 80,000 for AML with *AML1-ETO* (calculated from reported frequencies for these fusion genes in leukemia and the overall incidence rates of ALL and AML in the United States and Europe).

We tested this prediction by screening a large series of newborn cord blood samples by two independent assays: reverse transcriptase (RT)-PCR using nested primers and Taqman real-time quantitative PCR (15). By screening aliquots of the cDNA product of small-sized samples (1 ml/≈10^6 cells) and setting thresholds of positivity calculated (by Taqman) to be 10^{-5} or above, we reasoned that positive samples would have functional, in-frame fusion genes with clonally expanded cells. At the same time, this conservative approach should filter out any low-frequency translocation products that were nonfunctional because they were either out of frame or had not arisen in the appropriate stem cell types to endow clonal advantage. We assume that such "silent" events occur much more frequently than functional rearrangements. We took all possible steps to avoid contamination in RT-PCR tests (16) but, in addition, we sought to confirm the presence of fusion gene positivity at the single-cell level by interphase fluorescence *in situ* hybridization (FISH). The latter approach also allowed us to make two further predictions. First, that if functional, fusion genes should be present predominantly, if not exclusively, in the same cell types or lineages, as in the corresponding leukemia—i.e., *TEL-AML1* in the B cell lineage, *AML1-ETO* in myeloid cells. We assessed this prediction by cell enrichment and multicolor immunophenotyping/FISH analysis of single cells. Second, in contrast to ALL cells with *TEL-AML1* fusion that frequently have deletions of the normal *TEL* allele as a secondary genetic abnormality (17, 18), all putative preleukemic cells in cord blood with *TEL-AML1* fusion should retain the normal copy of the *TEL* allele on 12p.

Materials and Methods

Cord Blood Samples. Cord blood samples, derived from umbilical cord, were collected from two centers (London Cord Blood Bank and Bristol Transplantation Unit) as part of a program for clinical research and transplantation. Ethical approval for use in this project was obtained. One-milliliter aliquots of mononuclear cells previously stored in liquid nitrogen were thawed for use.

RNA Isolation and cDNA Synthesis. Total RNA was extracted by using TRIzol reagent (GIBCO/BRL) according to the instructions of the manufacturer. cDNA was generated from 4 μg of total RNA by using random hexamers in a final volume of 40 μl.

RT-PCR. Integrity of cDNA was assessed by amplification of β_2-microglobulin, and *TEL-AML1* and *AML1-ETO* fusions were

Abbreviations: ALL, acute lymphoblastic leukemia; AML, acute myeloblastic leukemia; RT, reverse transcriptase; FISH, fluorescence *in situ* hybridization.

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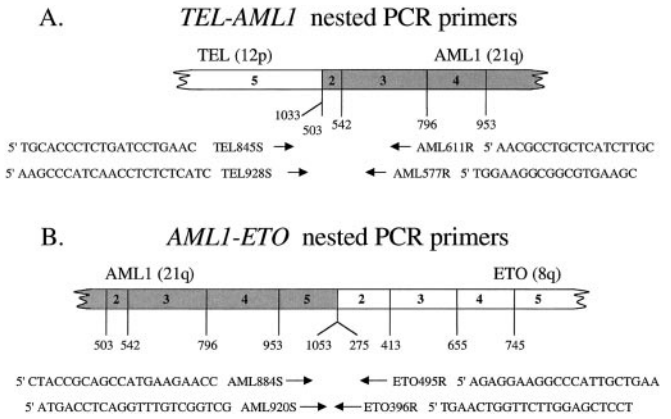

Fig. 1. Schematic diagram of fusion transcripts and the relative location of amplification primers (arrows) for *TEL-AML1* (*A*) and *AML1-ETO* (*B*).

amplified by nested RT-PCR (see Fig. 1). For *TEL-AML1*, 2 μl of cDNA was amplified in 50 μl of PCR mixture with 1.25 units HotStar *Taq*DNA polymerase (Qiagen, Chatsworth, CA) and 50 pmol of each primer, as described by the manufacturer. The reaction conditions were an initial activation step at 95°C for 15 min, followed by 35 cycles of 30 s at 94°C, 30 s at 60°C, 60 s at 72°C, with a 10-min final extension at 72°C. One microliter of first-round PCR products was reamplified under the conditions described above. For *AML1-ETO*, 2 μl of cDNA was also amplified essentially as above. PCR products were separated on 2.0% agarose gels and sequenced directly with the same primers used in the PCR.

Taqman PCR. The primers and probes for *TEL-AML1* and *AML1-ETO* were designed with PRIMER-EXPRESS software (Applied Biosystems) as follows: *TEL-AML1* forward, 5′-TGTCTCCCCGCCTGAAGA-3′; *TEL-AML1* reverse, 5′-TCGTGGACGTCTCTAGAAGGATT-3′; *TEL-AML1* detection probe, 5′-FAM CATTCCAAGTATGCATTCTGCTATTCTCCCAA-3′-TAM; *AML1-ETO* forward, 5′-CACCTACCACAGAGCCATCAAA-3′; *AML1-ETO* reverse, 5′-ATCCACAGGTGAGTCTGGCATT-3′; *AML1-ETO* detection probe, 5′-FAM CCCGAGAACCTCGAAATCGTACTGAGAAGC-3′-TAM. β_2-microglobulin as an internal control was amplified by using a commercially available kit (β_2-microglobulin primers and VIC-labeled probe, Applied Biosystems) at the same time. The PCR mix contained 1 μl cDNA template, 1× Taqman buffer A, 8% glycerol, 5 mM $MgCl_2$, 200 μM each of dATP, dCTP, and dGTP, 400 μM dUTP, 1.25 units AmpliTaq Gold DNA polymerase, 0.25 units AmpErase UNG, 300 nM each of the primers for *TEL-AML1* and 100 nM *TEL-AML1* probe or 900 nM each of the primers for *AML1-ETO* and 200 nM *AML1-ETO* probe in a total volume of 25 μl. Standard reactions were performed by using an Applied Biosystems PRISM 7700 Sequence Detection System. All experiments were carried out in triplicate.

Isolation of Cell Populations from Cord Blood. Subpopulations of cells were positively enriched by using either a flow sorter (Becton Dickinson, Facs Vantage) or standard immunomagnetic, MACS system protocol (Miltenyi Biotec, Bisley, Surrey, U.K.). For flow sorting, cells were labeled with one of the following fluorochrome-labeled primary antibodies of mouse IgG1 isotype (Dako): CD3 (UCHT1), CD19 (HD37), CD14 (UCHM-1), CD10 (SS2/36), and CD34 (QBend10). For immunomagnetic cell enrichment, the same unlabeled primary antibodies were used followed by magnetic labeling with rat anti-mouse IgG1 microbeads and enrichment on a positive section column. In the majority of cases the negative fraction of cells was also saved, subfractionated, and labeled with antibodies not used for the primary enrichment. Immunomagnetically selected cells were then used for combined or triple-color immunophenotype/FISH analysis.

Fluorescence Immunophenotype/FISH. Cells were fixed in acetone. Cells already labeled with antibody for enrichment (CD10, CD19) were further labeled with anti-mouse biotin followed by AvidinD^{AMCA} (for blue coloration). Cells enriched negatively as CD10$^-$ or CD19$^-$ were first stained with a CD13/CD33 mouse mAb mixture followed by biothylated anti-mouse Ig and AvidinD^{AMCA} or with mouse mAbs to human κ light chain or human λ light chain. After antibody staining, slides were hybridized with commercial (Vysis, Downes Grove, IL) *TEL-AML1* or *AML1-ETO* FISH probes by using the recommended protocol first verified as providing the expected signals on leukemic cell lines. Cells were analyzed with a Zeiss Axioskop fluorescence microscope equipped with epi-fluorescence and a triple band pass filter. Images were captured by using a charge-coupled device camera (Photometrics, Tucson, AZ) and SMART-CAPTURE software (Digital Scientific, Cambridge, U.K.). With the LSI TEL-AML1 ES dual-color translocation probe (Vysis), the expected signal pattern for a normal cell nucleus is two green and two red signals, corresponding to two normal copies each of *TEL* and *AML1*. This probe set contains a 350-kb probe for the 5′ end of TEL (exons 1–4) and a ≈500-kb probe covering the entire *AML1* gene. The expected signal pattern for a t(12;21) cell nucleus is one green (normal TEL), two red (normal *AML1* and residual *AML1*), and one red-green (yellow) fusion signal. The two red signals are of different sizes, the larger one corresponding to the normal *AML1* and the smaller to the 5′ portion of *AML1* that is translocated to the der(12).

Using the LSI AML1 ETO dual-color, dual fusion translocation probe (Vysis), the expected normal pattern for a normal cell nucleus is two green and two red signals corresponding to two normal copies each of *AML1* and *ETO*. In this probe mixture, both the *AML1* and *ETO* probes span the entire gene. In a cell containing the t(8;21), this probe set therefore results in two fusion signals, corresponding to both the *AML1-ETO* and the reciprocal *ETO-AML1* fusion, as well as one normal copy each of *AML1* and *ETO*. The expected signal pattern for a t(8;21) cell nucleus is one red (normal *ETO*), one green (normal *AML1*), and two red-green (yellow) fusion signals.

Results

Presence of Bona Fide Fusion Gene mRNA in Normal Cord Samples. Cord blood cDNA samples were screened by RT-PCR for *TEL-AML1* and *AML1-ETO* with the nested primers shown in Fig. 1. Of 567 samples screened for *TEL-AML1*, six (≈1%) were positive (Fig. 2*B*). A total of 496 samples were screened for *AML1-ETO* and one was positive (Fig. 3*B*). RT-PCR-generated amplicons were of the anticipated size for the major mRNA product of the in-frame fusion genes: 181 bp for *TEL-AML1* and 260 bp for *AML1-ETO*, as observed in leukemic cells. They were verified as bona fide by sequencing (Figs. 2*C* and 3*C*). All samples were further assessed on a separate aliquot of the original cDNA preparation with Taqman probes. There were seven positive results in Taqman assays and these were the same seven samples (six for *TEL-AML1*, one for *AML1-ETO*) that scored positively by RT-PCR. From the standardized Taqman curves obtained with *TEL-AML1* cell line (REH) and an *AML1-ETO* cell line (Kasumi 1), it was possible to determine the approximate frequency of cells in cord blood that were fusion gene mRNA positive (Fig. 4). This frequency was calculated to be between 10^{-3} and 10^{-4}.

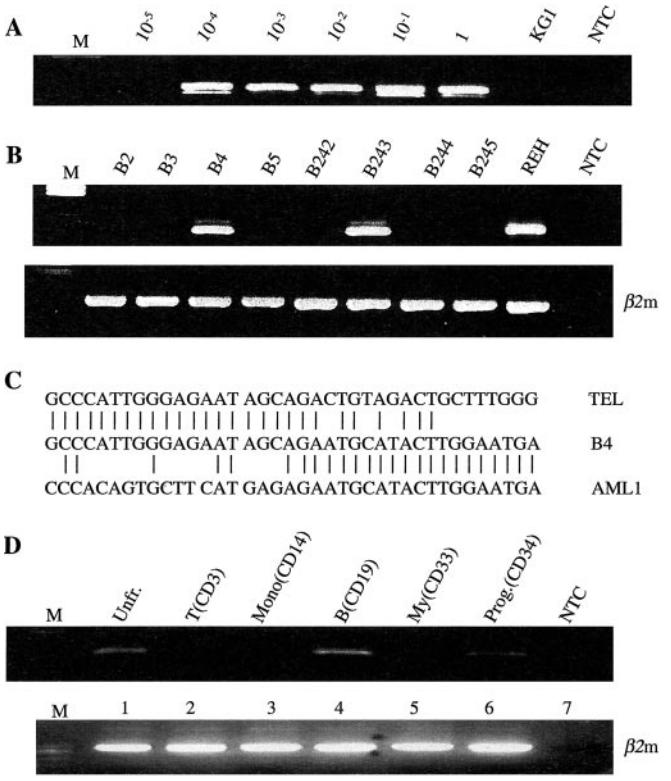

Fig. 2. Detection of *TEL-AML1* fusion transcripts in normal cord blood samples by nested RT-PCR. (*A*) *TEL-AML1*-positive control cells (REH leukemic cell line) were serially diluted in negative cells (KG1 leukemic cell line). NTC was no template control. Sensitivity of detection of *TEL-AML1* transcripts was 10^{-4}. (*B*) Representative screening results (second-round RT-PCR) for *TEL-AML1* fusion transcripts in cord blood. Two samples (B4, B243) were *TEL-AML1* positive and six samples (B2, B3, B5, B242, B244, and B245) were negative. β2m, β_2-microglobulin controls. (*C*) Representative fusion sequence of *TEL-AML1*-positive sample (B4). (*D*) RT-PCR analysis of different cell fractions from a *TEL-AML1*-positive cord blood (B4). β_2-microglobulin (β2m) used as positive control. NTC was no template control.

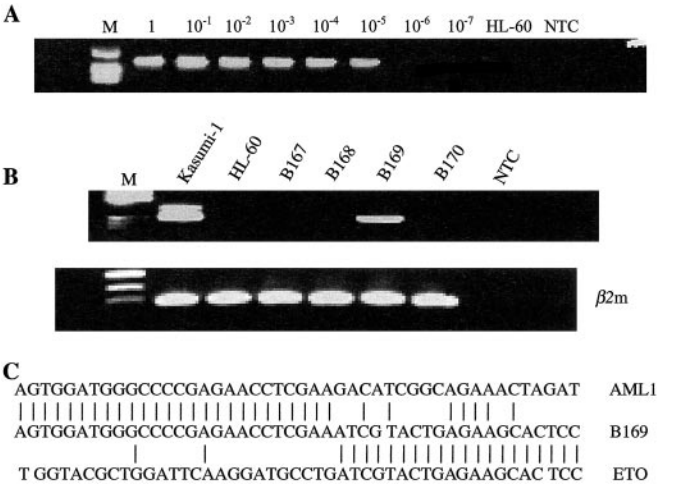

Fig. 3. Detection of *AML1-ETO* fusion transcripts in normal cord blood samples by nested RT-PCR. (*A*) *AML1-ETO*-positive control cells (Kasumi-1 leukemic cell line) were serially diluted in negative cells (HL-60 cell line). NTC was no template control. Sensitivity of detection of *AML1-ETO* transcripts was 10^{-5}. (*B*) Representative screening results (second-round RT-PCR) for *AML1-ETO* fusion transcripts. One cord blood sample (B169) was positive and three others shown here (B167, B168, B170) were negative. NTC was no template control. β2m, β_2-microglobulin controls. (*C*) Sequence of *AML1-ETO* derived from B169 cord blood cells.

Fusion Genes Are Present in Selective Cell Types and Lineages of Cord Blood. In one *TEL-AML1*-positive cord blood (B4), the original frozen unit was accessed to fractionate mononuclear cells into major cell types and lineages by using mAbs and FACS. RT-PCR and Taqman screening confirmed positivity in the unfractionated mononuclear cell population. $CD19^+$ (B cell lineage) and $CD34^+$ (stem/progenitor) cells were also positive (Fig. 2*D*). In contrast, there were no detectable amplicons in replicate (3–6 in total) samples of myeloid ($CD33^+$), T ($CD3^+$), or monocytic ($CD14^+$) cells. This pattern of selectivity is in accord with the early B lineage immunophenotype of leukemic cells with *TEL-AML1*.

In three additional *TEL-AML1*-positive cases and the one *AML1-ETO*-positive case, it was possible to retrieve sufficient viable cells from the remainder of the initial cord blood units to fractionate different cell types for analysis by FISH. Cells were separated with micromagnetic beads, antibody-fluorochrome stained, and then analyzed by FISH with dual-color probes for fusion genes.

The pattern of FISH staining usually observed in leukemic blasts from patients with ALL is as shown in Fig. 5*A*. The REH cell line is typical of t(12;21) positive leukemias in having lost the normal *TEL* allele, giving the typical FISH pattern of one TEL-AML1 fusion (yellow) and two AML1 (red) signals only. The simultaneous presence of both the yellow fusion signal and the two different-sized red *AML1* signals (representing the normal *AML1* allele and the small 5′ region of *AML1* translocated to chromosome 12) was taken as the most secure marker for *TEL-AML1* positivity in normal cord blood. Table 1 lists the data obtained, and Fig. 5 illustrates examples of cells observed.

In cord blood B128, both $CD10^+$ B cell precursors (Fig. 5*C*) and κ or λ Ig light chain-positive mature B cells ($CD10^-/CD19^+$ κ/λ^+) (Fig. 5*D*) were *TEL-AML1* fusion positive at a level of 0.25–0.33%. These cells also had two different-sized (red) *AML1* signals and retained the normal *TEL* allele (green signal in Fig. 5 *C* and *D*). No myeloid ($CD13^+/CD33^+$) cells were positive in 2,514 cells scored. Similarly, in sample B243, *TEL-AML1*-positive cells were present in the CD19-positive B cell lineage but absent from both T and myeloid cells (Table 1). In a control cord blood, negative for *TEL-AML1* transcripts, 3,199 cells in a CD10-positive fraction were scored and only one cell had both a fused red-green *TEL-AML1* signal plus two different-sized red (*AML1*) signals.

In cord blood B169 positive for *AML1-ETO*, the presence of fusion gene-positive cells at a level of $\approx$0.1% of $CD33^+/CD13^+$ myeloid cells was identified by immunophenotype/FISH analysis with each positive cell having two fusion signals—i.e., reciprocal translocations (Table 1; Fig. 6*C*). No $CD19^+$ B lymphoid cells were *AML1-ETO* positive. In a control cord blood sample, negative for *AML1-ETO* transcripts, there were no cells in 4,028 $CD33^+/CD13^+$ cells assessed with two fusion gene signals.

Discussion

These data confirm our prediction that cord blood cells in some individuals should express bona fide *TEL-AML1* and *AML1-ETO* fusion genes derived via chromosome translocation and that the number of such positive samples should exceed by at least 10- to 20-fold the cumulative risk of leukemia. The frequency of fusion gene-positive cord bloods we find (1%) to be approximately 100 times the cumulative frequency of overt, clinically diagnosed leukemia with the same fusion gene. We favor the view that the fusion gene-positive cells represent expanded clones of preleukemic cells that will remain pathologically and clinically silent or covert in the absence of additional, postnatal genetic hits. This interpretation is endorsed by murine

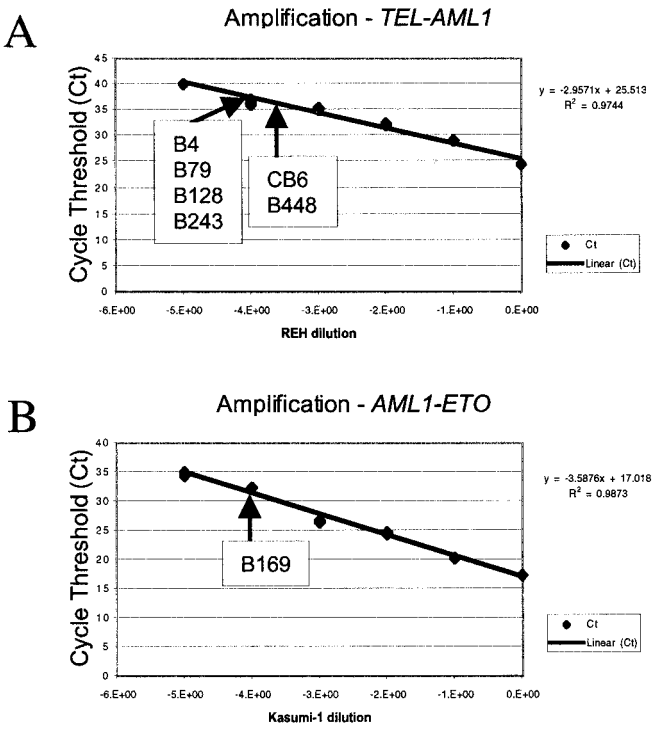

Fig. 4. Detection of *TEL-AML1* and *AML1-ETO* fusion transcripts in normal cord blood samples by real-time quantitative PCR (Taqman). Shown is a linear detection of *TEL-AML1* (*A*) and *AML1-ETO* (*B*) fusion transcripts (in REH and Kasumi-1 cell lines, respectively) over at least four and five logs, respectively. This assay could detect one *TEL-AML1*-positive cell in a background of 10,000 negative cells and one *AML1-ETO*-positive cell in a background of 100,000 negative cells. Cycle threshold values and positive cord bloods indicated with B or C numbers were used to calculate appropriate cell frequency (10^{-3} to 10^{-4}) from standard.

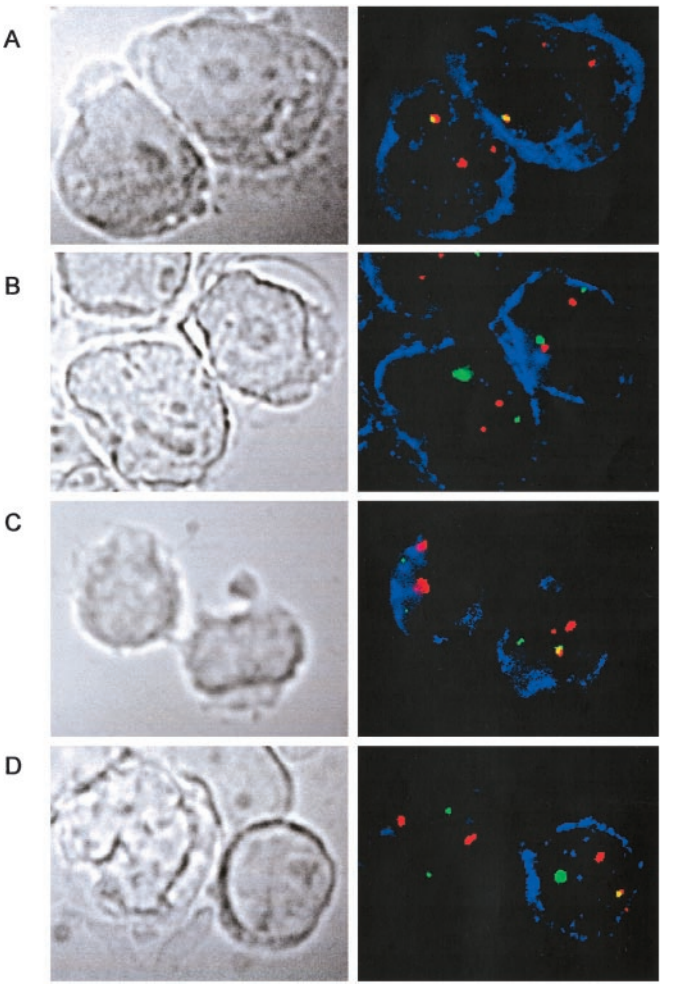

Fig. 5. Identification of *TEL-AML1* fusion gene-positive cells in cord blood (B128) by immunophenotype/FISH analysis. In each case the fluorescent signal corresponding to the *TEL* probe is green, the *AML1* probe is red, and the fused red-green signals corresponding to the *TEL-AML1* fusion appear yellow. The cells positive for the corresponding immunophenotype are stained blue. (*A*) Positive control leukemic cell line REH, stained for CD10. Both CD10-positive cells show one fusion and two separate red signals. The smaller red signal corresponds to the translocated 5′ portion of the disrupted *AML1* gene, and the larger red signal to the normal *AML1* allele. Note that the green signal is absent because of deletion of the normal TEL allele in this cell line (as in most cases of ALL with *TEL-AML1* fusion). (*B*) Negative control cell line Nalm 6, stained for CD10. Both CD10-positive cells show two green and two similar-size red signals, corresponding to two normal copies of TEL and AML1. (*C*) CD10-positive sorted cord blood (B128) cells. (*Right*) The cell shows one green, two different-sized red, and one (yellow) fusion signal, the expected signal configuration for a *TEL-AML1*-positive cell. The green (normal TEL) signal is present. (*Left*) The cell shows two green and two similar-sized red signals, corresponding to two normal copies of TEL and AML1. There is no fusion signal. (*D*) $CD10^-/CD34^-$ cord blood cells stained for Ig κ. The κ-positive blue cell (*Right*) shows one yellow fusion, one green, and two different-sized red signals, the expected pattern for a TEL-AML1-positive cell. The normal TEL signal is present in this cell. The other cell (κ negative) shows two normal copies of TEL and AML1. (Magnifications: ×1,500.)

model systems in which *TEL-AML1* or *AML1-ETO* transgenes are insufficient by themselves to produce leukemia (13, 14, 19) and by variable and protracted postnatal latency (up to 14 years) as revealed by studies on identical twins (6, 7, 9) and neonatal blood spots (9).

The possible preleukemic nature of the cells we identified with fusion genes in cord blood is supported by the finding that they are present at estimated frequencies (10^{-3} to 10^{-4}) that suggest significant clonal expansion. A preleukemic cell frequency in a positive cord blood of 10^{-3} to 10^{-4} mononuclear cells is credible as it corresponds to the estimated range of fusion gene-positive cells in the archived neonatal blood spots of children with *TEL-AML1*-positive ALL (9). Additionally, the fusion gene products detected are all in-frame and therefore potentially producing functional protein. We assume, but have not attempted to show, that these fusion genes are functionally distilled (i.e., by clonal selection) from a far higher rate of breaks occurring in the same genes, in a higher proportion (if not all) of developing fetuses, but that do not generate functional chimaeric protein or arise in the "appropriate" cell context for leukemogenesis.

The leukemic blasts in ALL with *TEL-AML1* fusion have B cell precursor phenotypes ($CD10^+/CD19^+$) in apparent differentiation arrest (20). Cells with *TEL-AML1* fusion in normal cord blood were also exclusively B cell lineage that would accord with a preleukemic (pre-ALL) status but included both precursors ($CD10^+$) and mature (κ/λ^+) B cells. This finding suggests that putative preleukemic clones with *TEL-AML1* fusion, in contrast to overtly leukemic cells, are still capable of terminal differentiation. The presence of both κ and λ light chain-positive cells might suggest that the *TEL-AML1* fusion was nonclonal, but the data are also compatible with the more likely possibility that the chromosomal translocation occurred in a single B lineage progenitor or stem cell whose progeny cells could selectively rearrange either κ or λ genes. These data accord with an origin of childhood B cell precursor/common ALL in a B lineage restricted stem cell. However, we cannot exclude that the

Table 1. Identification of *TEL-AML1* or *AML1-ETO* fusion by FISH

Cell fraction	No. of cells positive		(~% Positive)
Cord blood (B128) cells scored for *TEL-AML1* fusion*			
$CD10^+$ (B cell precursors)	6/2, 422		(0.25)
$CD10^-/CD19^+$ (pan mature B)	6/2, 665	Mature B	
κ^+ (mature B)	5/1, 118	16/4801	(0.33)
λ^+ (mature B)	5/1, 018		
$CD10^-/CD19^-/CD13^+/CD33^+$ (pan-myeloid)	0/2, 514		(<0.05)
Cord blood (B243) cells scored for *TEL-AML1* fusion*			
$CD19^+$ (pan B lineage cells)	13/5, 367		(~0.24)
$CD33^+/CD13^+$ (pan myeloid)	0/3, 386		(<0.03)
$CD3^+$ (pan T)	0/3, 446		(<0.03)
Cord blood (B169) cells scored for *AML1-ETO* fusion†			
$CD19^+$ (pan B lineage cells)	0/4, 887		(<0.02)
$CD13^+/CD33^+$ (pan myeloid)	5/4, 462		(0.11)
Negative control for *TEL-AML1* fusion*			
$CD10^+$ (B cell precursors)	1/3, 199		(<0.03)
Negative control for *AML1-ETO* fusion*			
$CD33^+$ $CD13^+$ (pan myeloid)	0/4, 028		(<0.02)

*Only cells with a fusion and an extra *AML1* signal (2R1G1F) were scored as *TEL-AML1* fusion positive. A third *TEL-AML1*-positive cord blood (B79) was also assessed by immunophenotype/FISH. In this case, although 1% of CD10-positive B cell precursors had a *TEL-AML1* fusion signal, the extra *AML1* signal was not seen. This is a recognized problem with the *TEL-AML1* ES probe (Vysis), which can be ascribed to the poorer hybridization efficiency for the smaller 5′ translocated *AML1* segment (C. Harrison, personal communication). However, it means that we cannot unequivocally distinguish true ''fusion positive'' red-green fused signals from coincidental overlap that is observed at low levels in normal controls.

†Only cells with two fusion signals (1R1G2F) were scored as *AML1-ETO* fusion positive.

chromosome translocation, t(12;21) originates in a multipotential (lymphomyeloid) stem cell with preferential clonal expansion in the B lymphoid lineage. This same caveat applies to the selectivity we observed of *AML1-ETO* fusion for myeloid cells in cord blood. Indeed, there is evidence that *AML1-ETO* may arise in lymphomyeloid stem cells (21).

As anticipated, all putative preleukemic cells in normal cord blood with *TEL-AML1* fusion retain the normal *TEL* allele. This finding accords with observations based on FISH and microsatellite loss of heterozygosity, indicating that *TEL* deletions are subclonal or secondary to *TEL-AML1* fusion in ALL (17, 18, 22) and are probably postnatal events (18).

There have been earlier reports of leukemia fusions in normal individuals but their functional significance is doubtful. A study suggesting that *MLL-AF4* fusion genes characteristic of infant ALL were detectable, by RT-PCR, in an exceptionally high frequency (≈25%) of newborn blood samples (23) was not subsequently confirmed by ourselves and others (24, 25). RT-PCR screening for infrequent cells or low copy number mRNA is vulnerable to problems with contamination. The test is generic and does not distinguish clone-specific products. In the current study, the use of isolated laboratory facilities, parallel RT-PCR, and Taqman screening followed by FISH effectively resolves this potential concern for *TEL-AML1* and *AML1-ETO* fusions. Other examples of leukemia fusion gene products in normal individuals appear to be real but functionally trivial. *BCR-ABL* mRNA, the fusion gene product of the Philadelphia chromosome associated with chronic myelogenous leukemia and ALL, is detectable in the blood of a high proportion of normal adults (26, 27). However, the extraordinarily low copy number in positive samples [5–20 copies of *BCR-ABL* mRNA per 5×10^7–10^8 cells or less than 10 positive cells per 10^8 (26, 27)] suggests that these rearrangements are almost certainly not arising in the stem cell compartment with consequent clonal expansion.

The t(14;18) *BCL2-IGH* translocation associated with follicular lymphoma is also detectable in the blood cells of a substantial fraction of normal adults, increasing in prevalence with age (28, 29). The functional relevance of these data to the natural history of lymphoma is unclear. In part, the remarkably common

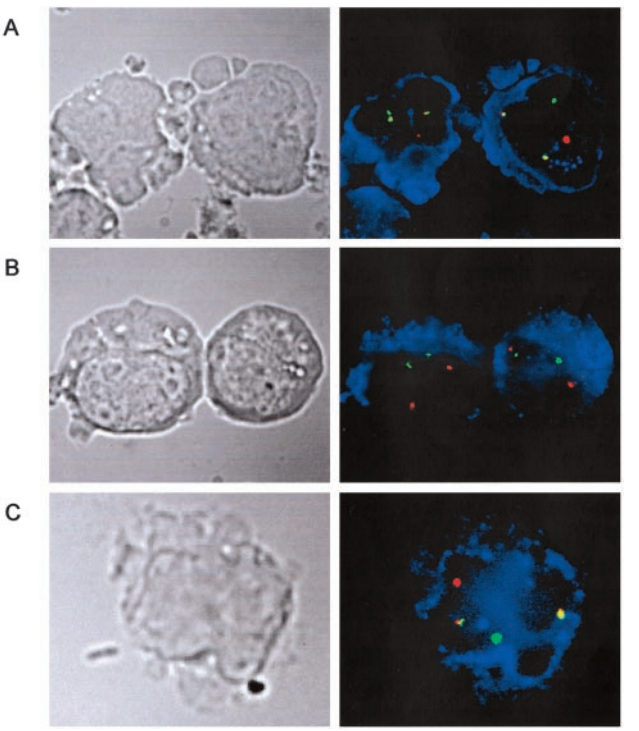

Fig. 6. Identification of AML1-ETO fusion gene-positive cells in cord blood (B169) by immunophenotype FISH analysis. In each case the fluorescent signal corresponding to the AML1 probe is red and the ETO probe is green. The cells positive for the respective immunophenotypes are stained blue. (*A*) Positive t(8;21) cell line Kasumi. Both CD13/CD33-positive cells show two red-green (yellow) fusion signals, corresponding to the AML1-ETO and ETO-AML1 gene fusions, in addition to the single green and red signals of the normal AML1 and ETO alleles. (*B*) t(8;21) negative control: Epstein–Barr virus-transformed normal blood lymphocytes stained for CD19. Both cells show two normal copies each of AML1 and ETO. (*C*) Cord blood sorted mononuclear cells stained for CD13/CD33. The CD13/CD33-positive cell shown has one red, one green, and two yellow fusion signals, consistent with the presence of the t(8;21). (Magnifications: ×1,500.)

occurrence of this particular rearrangement may reflect the intrinsic genomic instability of mature lymphocytes (30) and the propensity of immunoglobulin/T cell receptor recombinases to promote illegitimate recombinants (31).

The frequency of newborn individuals we find with cells expressing a bona fide leukemia fusion gene may have parallels in other pediatric cancers. The frequency we find for *TEL-AML1* is approximately the same (≈1%, 100 times the disease incidence rate) as that recorded for putative premalignant lesions, defined at autopsy by histopathology, resembling neuroblastoma (32), and Wilm's tumor (nephrogenetic rests) (33). This finding suggests that several common pediatric cancers may be initiated at a high rate but with a low risk (≈1 in 100) of penetrance to full malignancy. These data accord with the classical "two-hit" model of Knudson for noninheritable cancers in childhood (11), but additionally provide, perhaps surprising, insight into how often premalignant clones arise during normal fetal development. If our data were to apply to all subtypes of pediatric leukemia, then it follows that around 5% or 1 in 20 children are born with preleukemic clones—i.e., 100 times the cumulative frequency of 1 in 2,000 for leukemia before the age of 15 years (34). An important corollary is that major rate-limiting events in the emergence of pediatric cancer would appear to be later-stage promotional exposures and/or associated secondary genetic alterations.

There are other implications for etiological mechanisms in childhood leukemia. Genomic sequences of *TEL-AML1* and *AML1-ETO* fusions suggest that they are probably generated by error-prone nonhomologous end-joining (4, 35), the prevalent normal repair process for double-stranded DNA breaks in mammalian chromosomes (36). Whatever exogenous or endogenous DNA damaging exposures actually initiate these chromosome translocations in the fetus, they are likely to be relatively common.

These insights derived from cord blood may also have important practical implications for clinical management of children with leukemia. Presumptive preleukemic cells might well coexist with overt leukemic blasts at diagnosis, survive chemotherapy (21), and occasionally spawn later relapses (22). If so, then this work has implications for PCR-based monitoring of minimal residual disease and for our understanding of the biological basis of cure and relapse in leukemia. Additionally, these data raise some safety concerns about the use of stored cord blood for transplantation purposes, particularly in an autologous setting for leukemia patients (37).

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