Alu elements mediate *MYB* gene tandem duplication in human T-ALL

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Recent studies have demonstrated that the *MYB* oncogene is frequently duplicated in human T cell acute lymphoblastic leukemia (T-ALL). We find that the human *MYB* locus is flanked by 257-bp Alu repeats and that the duplication is mediated somatically by homologous recombination between the flanking Alu elements on sister chromatids. Nested long-range PCR analysis indicated a low frequency of homologous recombination leading to *MYB* tandem duplication in the peripheral blood mononuclear cells of ~50% of healthy individuals, none of whom had a *MYB* duplication in the germline. We conclude that Alu-mediated *MYB* tandem duplication occurs at low frequency during normal thymocyte development and is clonally selected during the molecular pathogenesis of human T-ALL.

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T cell acute lymphoblastic leukemia (T-ALL) is a thymocyte malignancy that accounts for 10–15% of childhood and 25% of adult ALL cases. Molecular studies of recurrent chromosomal translocations in T-ALL have indicated a central role for the aberrant expression of transcription factors in the pathobiology of this disease. Dysregulated expression of *TAL1*, *MYC*, *HOX11*, and *LMO2*, for example, can lead to abnormal proliferation and differentiation arrest of T-lymphoid progenitors (1). More recently, activating mutations of the *NOTCH1* gene were identified in >50% of human T-ALL cases (2). *NOTCH1* encodes a transmembrane receptor with important functions in T cell development; activation of the receptor leads to proteolytic cleavage and release of the NOTCH intracellular domain, which transits to the nucleus and functions as a component of a transcriptional complex that regulates the expression of *MYC* and other key target genes (3–5).

The *MYB* transcription factor is the cellular counterpart of the *v-Myb* oncogene of the acutely transforming avian myeloblastosis virus, which causes a rapidly fatal monoblastic leukemia in chickens (6, 7). *Myb* is essential for hematopoietic, as well as T cell, development (8, 9), and when overexpressed in thymocytes, *v-Myb* causes T-ALL (10). Accordingly, retroviral insertion and transcriptional activation of the *Myb* locus represents one of the most frequent accelerating events in mouse models of T-ALL (11) (http://rtcgd.abcc.ncifcrf.gov/). In human cancers, *MYB* is known to be amplified in 2 colon cancer cell lines, 4 glioblastoma cell lines, and 29% of *BRCA1*-mutated primary breast cancer samples (12–14). *MYB* duplication and translocation was very recently demonstrated in human T-ALL (15, 16). We show that the most important mechanism underlying *MYB* copy number alteration in T-ALL occurs somatically by homologous recombination between Alu elements, providing a

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means by which an evolving T-ALL clone develops an increased dosage of *MYB* during leukemic transformation.

RESULTS AND DISCUSSION

The *MYB* gene is tandemly duplicated in human T-ALL

While conducting high-resolution microarray-based comparative genomic hybridization (array CGH) analysis of full-complexity DNA derived from 17 established T-ALL cell lines, as well as bone marrow samples from 8 cases of newly diagnosed T-ALL, we observed a highly localized increase in copy number within the 6q23 region, which encompasses the *MYB* gene and part of the nearby downstream *AHI1* gene in 6 cell lines and 2 clinical samples (Fig. 1, A–C; and Figs. S1 and S2, available at http://www.jem.org/cgi/content/full/jem.20071637/DC1).

To determine the mechanisms of increased *MYB* copy number in T-ALL, we used fluorescent in situ hybridization (FISH) with a commercially available probe for *MYB*. 2 of the 17 T-ALL cell lines examined showed increased copies of *MYB* relative to the copy number of the chromosome 6 centromere (Molt4 and Molt13; Table I and Fig. S3, available at http://www.jem.org/cgi/content/full/jem.20071637/DC1), representing translocations of chromosome 6q regions to other chromosomes. These cell lines did not show the localized increases in *MYB* copy number found with array CGH (Fig. 1 B, samples 7 and 8). In fact, none of the cell lines with a discrete increase in *MYB* copy number had abnormalities affecting this locus by interphase FISH (Fig. S3). Thus, we postulated that the localized increase of *MYB* copy number observed by array CGH must affect a region adjacent to one

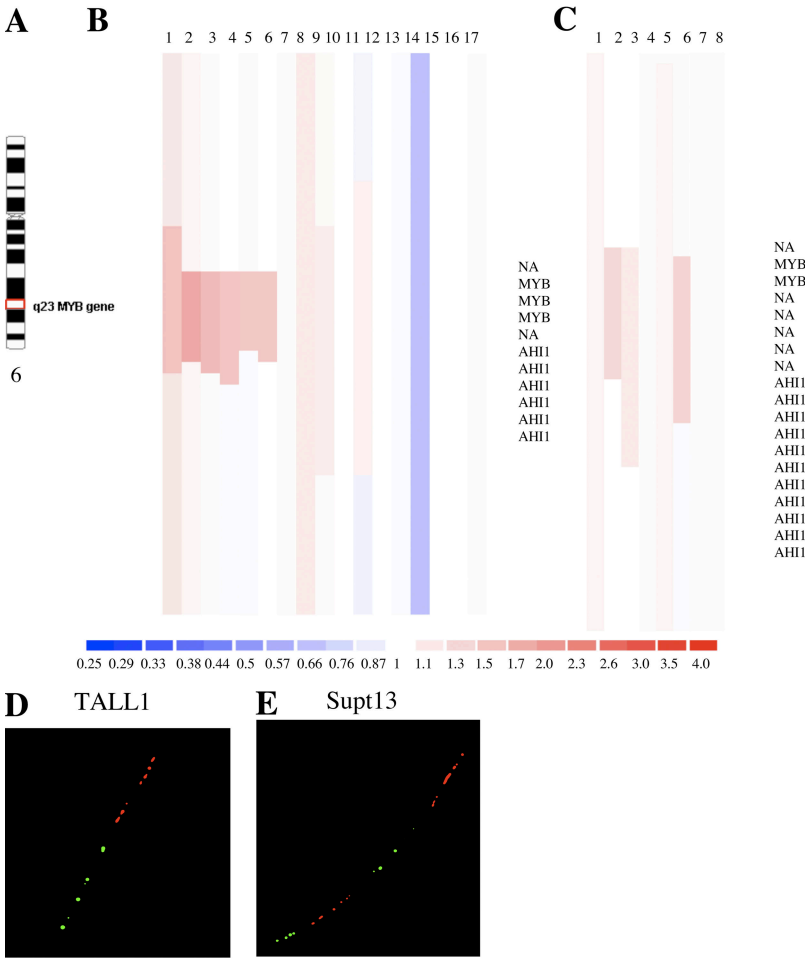

Figure 1. The MYB gene is tandemly duplicated in human T-ALL cell lines and patient samples. (A) Ideogram of chromosome 6 showing location of the *MYB* gene on q23. (B) Array CGH performed on DNAs from 17 T-ALL cell lines with Human Genome CGH 44K Microarrays (Agilent). A localized region on 6q23 surrounding the *MYB* locus is shown to have increased copy number in six of the cell lines. A 2.3-Mb region of chromosome 6 is shown. The top is centromeric, and the bottom is telomeric. Part of the *AHI1* gene directly downstream of *MYB* is also amplified. NA indicates a probe in an intergenic region. Red indicates increased copy, blue indicates decreased copy, and the intensity of the color reflects the level of increase or decrease. (C) DNAs from leukemic cells in the diagnostic bone marrow of eight T-ALL patients were similarly analyzed by array CGH using Human Genome CGH 244K Microarrays, with an increased *MYB* copy number identified in two cases. A 500-kb region of chromosome 6 is shown. (D) Fiber-FISH on a T-ALL (TALL1) cell line with a diploid *MYB* copy number. The fosmid encompassing most of the *MYB* gene is labeled in green; a fosmid immediately 3′ of the *MYB* coding sequence is labeled in red. (E) Fiber-FISH on the Supt13 cell line showing a duplication of both fosmids spanning the entire *MYB* locus oriented in tandem on the same DNA fiber.

Table I. Summary of results for 17 T-ALL cell lines

Number[a]	Cell line	MYB status by array CGH	MYB status by MLPA	Long-range PCR	Breakpoint subtype[b,c]	Interphase FISH	Fiber-FISH[c]
1	ALL-SIL	Increased copy	Increased copy	Negative-larger region amplified	NA	No increase relative to centromere	Tandem duplication
2	BE13	Increased copy	Increased copy	Positive	1,2,3	No increase relative to centromere	Tandem duplication
3	CEM	Increased copy	Increased copy	Positive	6	No increase relative to centromere	Tandem duplication
4	RPMI8402	Increased copy	Increased copy	Positive	3,4,5	No increase relative to centromere	ND
5	Supt13	Increased copy	Increased copy	Positive	2,3	No increase relative to centromere	Tandem duplication
6	DU528	Increased copy	Increased copy	Positive	2,3,4,5	No increase relative to centromere	ND
7	Molt4	Increased copy	Increased copy	Negative	NA	Increased *MYB* relative to centromere	ND
8	Molt13	Increased copy	Increased copy	Negative	NA	Increased *MYB* relative to centromere	Tandem duplication
9	TALL1	Inconclusive	Deletion	Positive	4	*MYB* Translocation	Normal
10	PF382	No increase	No increase	Negative	NA	ND	ND
11	Supt7	No increase	No increase	Positive	ND	ND	ND
12	Molt16	No increase	Increased copy	Negative	NA	No increase relative to centromere	ND
13	DND41	No increase	Inconclusive	Positive	1,2,3,4,5	No increase relative to centromere	ND
14	Loucy	6q deletion	Deletion	Negative	NA	Deletion	ND
15	Jurkat	No increase	No increase	Negative	NA	No increase relative to centromere	Normal
16	Koptk1	No increase	No increase	Positive	3,4,5	No increase relative to centromere	ND
17	Supt11	No increase	No increase	Negative	NA	No increase relative to centromere	ND

[a]Numbers correspond to labels in Fig. S1 and Fig. S4 B.
[b]Based on subtypes shown in Fig. 1 D.
[c]ND, not done; NA, not applicable.

of the normal *MYB* alleles, resulting in a merged signal by FISH on interphase nuclei and on metaphase chromosome spreads. We therefore used fiber-FISH to hybridize 5′ and 3′ *MYB* fosmid probes onto stretched DNA fibers from interphase cells of seven different T-ALL cell lines. This strategy demonstrated that *MYB* is tandemly duplicated on one allele in all five human T-ALL cell lines analyzed with array CGH findings of localized increases in *MYB* copy number (Fig. 1, D and E; and Table I). Thus, the increased *MYB* copy number in T-ALL that we and others (15, 16) have observed using array CGH is caused by tandem duplication.

Tandem duplication of *MYB* is mediated by Alu elements flanking the *MYB* gene

To clone the breakpoints between the duplicated copies of *MYB* in T-ALL and define the mechanism underlying *MYB* tandem duplication, we performed long-range PCR. From the duplicated genomic region on chromosome 6, as defined by others (15, 16), and from our own array CGH data on T-ALL cell lines and patient samples (Tables I and II and Fig. 1), we designed a forward primer 54 kb 3′ of the end of the *MYB* coding sequence and a reverse primer at the 5′ end of the *MYB* gene (primers 1 and 6; Fig. 2 A). This strategy generated an amplified band of ∼10 kb from T-ALL cell lines (Fig. 2 A). Two more rounds of nested PCR with forward primers progressively 3′ of the *MYB* gene and reverse primers further 5′ of the *MYB* gene yielded a 1-kb product that was sequenced (primers 3, 4, 5, 7, and 8; Fig. 2 A). Comparison of the sequence of this product with the normal human genome sequence identified a junction between the duplicated copies of *MYB*. Analysis of 15 T-ALL samples and cell lines showed that this junction always occurred within a 257-bp region of sequence homology that occurs both 3.5 kb 5′ and 73.5 kb 3′ of *MYB*. Analysis of this sequence using RepeatMasker

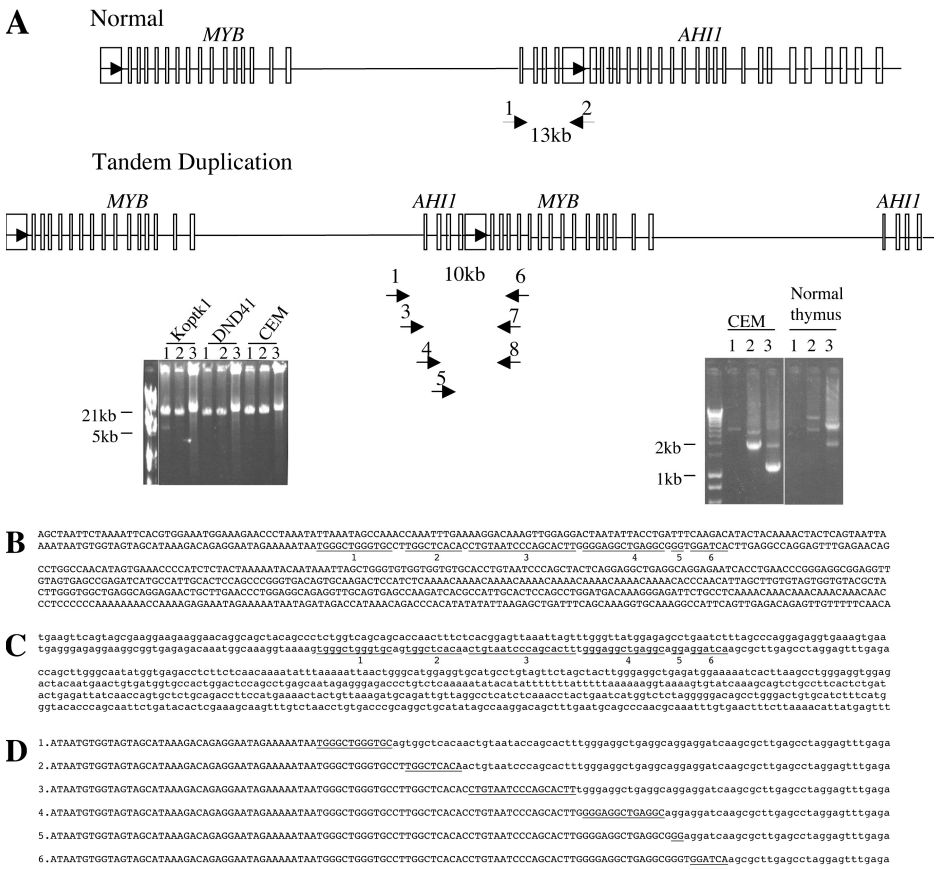

Figure 2. *MYB* duplication is mediated by *Alu* elements on chromosome 6. (A) Detection of the *MYB* tandem duplication by long-range PCR. Primers were designed based on the fosmids used in the fiber-FISH experiments (Fig. S3). Primers 1, 6, and 7 were used in long-range PCR to detect the *MYB* duplication. Primer 2 is a control primer for long-range PCR. Primers 3, 4, 5, and 8 are nested primers used to generate a smaller product that was sequenced. Arrows inside boxes indicate *Alu* elements and their orientation. Insets show PCR results on representative cell lines. (left) Primers 1 and 6 were used in reaction 1; primers 1 and 7 were used in reaction 2; and primers 1 and 2 were used in reaction 3. (right) After three rounds of nested PCR, a band of 1 kb (reaction 3) was obtained in the CEM cell line, but not in normal thymocyte DNA. Primers 3 and 8 were used in reaction 1; primers 4 and 8 in reaction 2; and primers 5 and 8 in reaction 3. (B) Sequence in upper case is 116 kb downstream of *MYB* in the *AHI1* locus. (C) Sequence in lower case is 3.5 kb upstream of *MYB*. (D) Sequences 1–5 represent the 5 different junctions between the 2 copies of *MYB* detected in T-ALL cell lines and primary samples. One additional breakpoint (sequence 6) was identified in normal lymphoblastoid cell line DNA. Underlined sequences represent the proposed crossover region. Fig. S3 is available at http://www.jem.org/cgi/content/full/jem.20071637/DC1.

(http://www.repeatmasker.org) revealed that it constitutes an Alu element. Alu repeats are short, repetitive DNA sequences of <500 bp that are estimated to account for 10% of the human genome (17). The Alu repeats on either side of the *MYB* gene are 76% identical, implicating homologous recombination as the underlying mechanism of the tandem duplication (Fig. 2, B and C) (18). Sequence differences between the upstream and downstream Alu elements enabled us to group the junctions of T-ALL cell lines into five classes (Fig. 2 D) (1–5). In each class, the downstream Alu element had merged directly with the upstream Alu region of the duplicated gene in a manner that is most consistent with a synthesis-dependent strand annealing (SDSA) event. In brief, the sequences from the downstream Alu element from one sister chromatid invade the upstream Alu element on the other sister, accompanied by DNA synthesis that proceeds past the downstream homologous sequence. The event is completed by strand annealing of the two ends that now contain identical sequence information (Fig. 3). We demonstrate that Alu elements on either side of the *MYB* gene mediate its somatic tandem duplication in a significant subset of T-ALL cell lines and patients. Previous reports have identified a larger region of duplication in some samples, which encompasses more than the *MYB* locus (15, 16). We have also seen that the duplicated region can be larger, and, in these cases, we cannot detect the duplication using our long-range PCR method (e.g., cell line 1 in Fig. 1 B). In these cases, the duplication either occurs by a different mechanism or is mediated by another region of homology.

As shown in Table I, we detected *MYB* tandem duplication by long-range PCR, but not by multiplex ligation-dependent probe amplification (MLPA) or array CGH in 4 T-ALL cell lines: TALL1, Supt7, DND41, and Koptk1. We also detected *MYB* tandem duplication by long-range PCR in 11 of 25 (44%) primary T-ALL samples (Table II), which

is a higher frequency than that found by MLPA in these samples. Thus, nested long-range PCR detects the tandem duplication in a minority of cells, whereas MLPA and array CGH are quantitative assays that detect *MYB* tandem duplication only when it occurs in a majority of the cells. Collectively, our results indicate that *MYB* tandem duplication occurs relatively often in T-ALL patients, but is clonally selected in only approximately one fifth of the cases during evolution of the disease, presumably because some malignant clones have increased *MYB* copies that have translocated to other chromosomes (as in Molt4 and Molt13; Table I and Fig. S3, available at http://www.jem.org/cgi/content/full/jem.20071637/DC1) or already express sufficient MYB protein levels by virtue of their differentiation state to optimally synergize with other oncogenic events, such as mutation of the *NOTCH1* gene (Fig. S4). This conclusion is supported by MLPA analysis showing the absence of *MYB* tandem duplication in remission samples from two patients whose malignant clones were positive for *MYB* duplication at diagnosis (Table II).

MYB tandem duplication in normal lymphocytes and peripheral blood cells

To determine whether *MYB* duplication occurs in normal lymphocytes, we performed long-range PCR on 96 DNA samples extracted from lymphoblastoid cell lines from the HapMap panel of individuals. We detected a band representing *MYB* tandem duplication in 45 of 96 samples (46.9%), and sequencing of all the products revealed that the breakpoints in normal samples were of the 5 types previously identified in T-ALL cell lines (Fig. 2 D), with the identification of one additional breakpoint (no. 6). Further analysis with MLPA did not detect an increase in *MYB* copy number in any of the 36 HapMap lymphoblastoid cell lines that were analyzed, implying that the duplication occurs infrequently, if at all, as a germline copy number variation, but rather is a somatic event affecting a small fraction of normal lymphocytes. To determine whether RAG expression is necessary for Alu-mediated *MYB* duplication, we have analyzed the AML cell lines HL60 and Meg01. We have detected the *MYB* gene duplication in these two lines, and sequencing of the PCR products revealed

Table II. Summary of results for 25 T-ALL patient samples

Number[a]	MYB status by array CGH[b]	MYB status by MLPA	Long-Range PCR for tandem duplication	Breakpoint subtype[c]
1	No increase	Deletion	Positive	1, 2, 3, 4
2	Increased copy	Increased copy	Negative	NA
3	Inconclusive	No increase	Positive	ND
4	No increase	No increase	Negative	NA
5	No increase	No increase	Positive	ND
6	Increased copy	Increased copy (not in remission)	Negative	NA
7	No increase	No increase	Negative	NA
8	No increase	No increase	Positive	ND
9	ND	No increase	Negative	NA
10	ND	No increase	Positive	1, 2, 3, 4
11	ND	No increase	Negative	NA
12	ND	No increase	Positive	5
13	ND	No increase	Positive	1, 2, 3, 4, 5
14	ND	Increased copy (not in remission)	Positive	1, 2, 3, 4
15	ND	No increase	Negative	NA
16	ND	No increase	Negative	NA
17	ND	No increase	Positive	1,2,3
18	ND	ND	Negative	NA
19	ND	ND	Positive	ND
20	ND	ND	Negative	NA
21	ND	No increase	Negative	NA
22	ND	ND	Positive	1, 2, 3, 4, 5
23	ND	No increase	Negative	NA
24	ND	Increased copy	Negative	NA
25	ND	Increased copy	Negative	NA

[a]Samples 1–8 correspond to samples 1–8 in Fig. S4 C.
[b]ND, not done; NA, not applicable.
[c]Based on subtypes shown in Fig. 1 D.

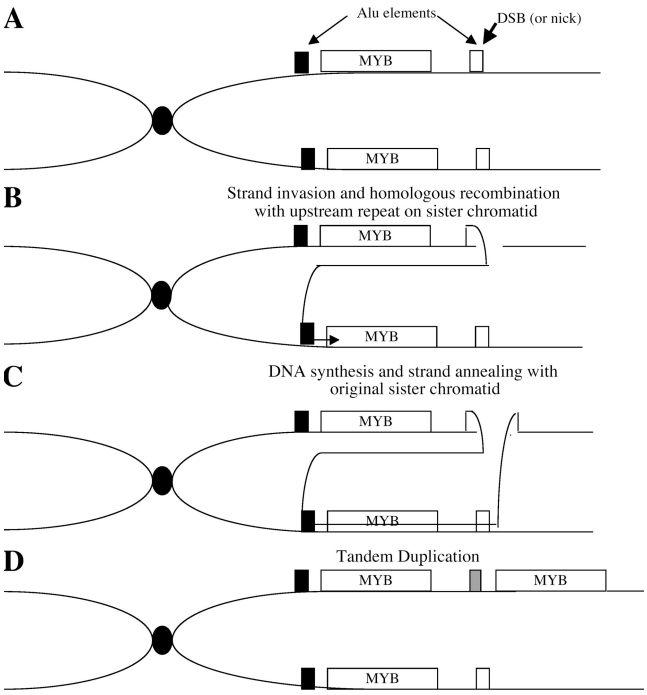

Figure 3. Proposed mechanism of *MYB* tandem duplication. (A) A DNA double-strand break occurs in (or near) the Alu element downstream of *MYB*. (B) The broken DNA strand invades the sister chromatid and pairs with the Alu element upstream of *MYB*. (C) DNA repair synthesis continues through the *MYB* gene, and the newly synthesized strand anneals to the broken chromatid by strand annealing. (D) The result is duplication of the sequence between the Alu elements. The sequence of the Alu element between the two copies of *MYB* (gray box) is a hybrid of the two repeats on either side of *MYB*, depending on where the strand anneals.

that the duplication was Alu-mediated, as in the T-ALL cell lines and samples. Therefore, RAG expression is not required for Alu-mediated *MYB* gene duplication.

We also analyzed 10 peripheral blood and 37 buccal swab samples from healthy individuals for the *MYB* tandem duplication by long-range PCR and MLPA. We detected the duplication in 2 of 10 blood samples and none of 37 buccal samples by long-range PCR. However, none of the blood or buccal samples was positive for *MYB* tandem duplication by MLPA. These results demonstrate that *MYB* tandem duplication occurs spontaneously in normal blood cells and is not an artifact of generating lymphoblastoid cell lines from normal cells. They also indicate that circulating blood mononuclear cells are more prone than epithelial cells to undergo *MYB* tandem duplication through Alu-mediated homologous recombination (Fisher's exact test, $P = 0.042$). Blood cells may be more likely to undergo this recombination event because they are more prone to double-strand breaks that initiate the Alu-mediated recombination.

Recent studies in mice have demonstrated that distinct levels of MYB are required at different stages of hematopoietic development for differentiation and proliferation (19, 20). Therefore, it is not surprising that a relatively modest increase in *MYB* expression, produced as a result of tandem duplication or translocation of a single copy, would be clonally selected in some T-ALLs during their molecular pathogenesis. Intriguingly, in several of our primary T-ALL samples with evidence by nested long-range PCR of rare cells with *MYB* tandem duplication, this subset of cells did not have a growth advantage, and the *MYB* copy number of the majority of cells was normal by CGH and MLPA. Thus, if a particular T-ALL clone already expresses sufficiently high levels of MYB for transformation because of its differentiation state, then the increased levels provided by *MYB* duplication may not provide an advantage for clonal selection.

We and others (15, 16) have observed that *MYB* duplication occurs often in T-ALL cases with activating mutations in *NOTCH1*. Knockout studies in the mouse, as well as *MYB* small interfering RNA experiments in T-ALL cell lines, demonstrated that *MYB* plays an important role in T cell differentiation (15, 21–23). Furthermore, γ-secretase inhibitors were shown to synergize with *MYB* small interfering RNA to inhibit T-ALL cell growth (15). These results are consistent with studies in the zebrafish showing that Notch and Myb act in parallel pathways to specify hematopoietic stem cell fate in the AGM (24) and indicate that *NOTCH* activation and *MYB* duplication synergize not only in normal blood cell development, but also in malignant T cell transformation.

Our study introduces a new mechanism by which an evolving T-ALL clone can increase the dosage of a critical oncogene during leukemic transformation. Homologous recombination between Alu elements in meiotic germ cells leading to deletions and duplications are responsible for several genetic diseases, including glycogen storage disease type II, Lesch-Nyhan syndrome, and Ehler-Danlos syndrome (25). Alu elements have also been implicated in mediating somatic genetic alterations in cancer, such as *MLL* translocations and partial tandem duplications in acute myelogenous leukemia (26, 27), *BRCA1* gene deletions in breast cancer (28), and *TRE* rearrangement in Ewing's sarcoma (29). Alu elements have also been found near the *LMO2* and *LCK* chromosomal breakpoints in T-ALL (30, 31), suggesting that they may also play role in these chromosomal translocations. Because Alu elements make up a large percentage of the human genome, it is likely that the copy number of many other oncogenes might be increased, or tumor suppressors deleted, through the recombination mechanism that we describe. The emergence of technologies for global comparative genomic hybridization and single-nucleotide polymorphism analysis in human cancers should enable efficient detection of these pathogenic copy number alterations, especially when the analyses are paired with analysis of normal germline DNA from the same individual.

MATERIALS AND METHODS

Patient materials. Cryopreserved lymphoblasts or lymphoblast lysates ALL were collected with informed consent and institutional review board approval from patients with newly diagnosed T-ALL who were treated with Dana-Farber Cancer Institute Acute Lymphoblastic Leukemia Consortium protocols 95-001 or 00-001. Genomic DNA was extracted with the PUREGENE kit according to the manufacturer's protocol (Gentra Systems).

Microarray-based CGH. Genomic DNA was fragmented and random-prime labeled, as previously described (32). Labeled DNAs were hybridized to microarrays containing 44K (cell lines) or 244K (primary samples) 60-mer oligonucleotides (Agilent Technologies), as previously described (33). Microarray data have been deposited in the GEO database under accession no. GSE7615.

FISH. FISH was performed with a commercially available probe mix containing a *MYB* (6q23) probe labeled with spectrum aqua and a centromeric probe for chromosome 6 labeled with spectrum orange (Vysis, Inc.). Slides and probe were co-denatured at 75°C, hybridized overnight at 37°C, washed, counterstained with 4',6-diamidino-2-phenylindole, and analyzed under a fluorescence microscope (Carl Zeiss, Inc.). For each cell line, a minimum of 100 nuclei and any identifiable metaphases were scored. The orange chromosome 6 signals and the aqua LSI *MYB* signals were counted simultaneously in each nucleus/metaphase. *MYB* was considered to be amplified if the cells showed more aqua signals than orange signals.

Fiber-FISH. Cells were lysed overnight using PUREGENE Cell Lysis Solution (Gentra Systems). The DNA was stretched on poly-L-lysine–coated slides, and the DNA fibers were fixed in 100% ethanol. To generate FISH probes, fosmids G248P89100B2 and G248P89828A1 were labeled with digoxigenin and biotin, respectively, using BioPrime Array CGH Labeling Module (Invitrogen). DNA fibers and labeled FISH probes were co-denatured for 3 min at 95°C and hybridized overnight in a humidified chamber at 37°C. The haptens were detected using anti–digoxigenin-fluorescein/Fab fragments (Roche) and Strepavidin/Alexa Fluor 594 conjugate (Invitrogen). Slides were analyzed under a fluorescence microscope (model BX51AI; Olympus). A normal allele showed green dots followed by red dots. A tandem duplication was seen as a direct repeat of the normal signal pattern.

MLPA. MLPA probe mix containing all probes was added to 150 ng denatured genomic DNA. A universal primer pair was used to amplify all ligated probes in a multiplex PCR assay. The PCR product was analyzed by sequence-type electrophoresis and samples were compared with two control samples. A difference in relative peak area indicated a copy number change of the DNA sequence targeted by the probe. A probe was considered amplified if the ratio sample/control was >1.3.

Long-range PCR. Reactions were performed with 500 ng of genomic DNA and the LA PCR kit version 2.1 (Takara Mirus Bio) according to the manufacturer's specifications. Primer sequences are available upon request.

Online supplemental material. Figs. S1 and S2 show array CGH data on T-ALL cell lines and patient samples analyzed by CGH Analytics (Agilent Technologies). Fig. S3 shows MYB FISH on T-ALL cell lines. Lines with tandemly duplicated *MYB* do not show increased copies of *MYB* by this method; however, two T-ALL cell lines with extra copies of MYB dispersed to other chromosomes were identified. Fig. S4 shows that *MYB* duplication results in increased levels of MYB RNA and protein levels in T-ALL cell lines. The online version of this article is available at http://www.jem.org/cgi/content/full/jem.20071637/DC1.

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