Proc. Natl. Acad. Sci. USA
Vol. 94, pp. 3899–3902, April 1997
Genetics

The partial tandem duplication of *ALL1* in acute myeloid leukemia with normal cytogenetics or trisomy 11 is restricted to one chromosome

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Communicated by Carlo M. Croce, Thomas Jefferson University, Philadelphia, PA, February 11, 1997 (received for review January 7, 1997)

ABSTRACT **The molecular defects responsible for tumorigenesis in adult *de novo* acute myeloid leukemia (AML) with a normal karyotype or an additional copy of one chromosome (i.e., trisomy) remain largely unknown. We recently discovered that approximately 90% of adult patients with *de novo* AML and trisomy 11 (+11) as a sole abnormality and 11% of adult patients with *de novo* AML and normal cytogenetics carry a molecular rearrangement of the *ALL1* (*MLL, HRX,* or *HTRX*) gene. The rearranged *ALL1* gene has been shown to result from the direct tandem duplication of a portion of *ALL1* itself. To better understand the underlying mechanisms of leukemogenesis, we asked whether in cytogenetically normal cases one or both chromosomes carry the mutated allele and whether in trisomic cases the mutation is present in one, two, or three chromosomes. Herein we show that in cytogenetically normal cases of AML and in cases with +11 as a sole cytogenetic abnormality, only one chromosome contains the mutated *ALL1* allele. Thus a single mutated *ALL1* allele with the partial tandem duplication is sufficient for *ALL1*-associated leukemogenesis, irrespective of the number of normal genes present. The frequently occurring specific association of +11 and *ALL1* gene mutation in the leukemic clone remains unexplained.**

Reciprocal chromosomal translocations or inversions have led to the identification of the majority of genetic defects known to contribute to malignant transformation in acute myeloid leukemia (AML) (1, 2). However, normal diploid chromosomes or clonal evidence for trisomy (+) of chromosome 21 (+21), 8 (+8), or 11 (+11) as a sole abnormality on cytogenetic analysis of leukemic blasts account for approximately 45% of AML cases (3). In the majority of these cases, the specific genetic mutations responsible for AML have not been identified. Recently, we discovered the first consistent molecular defect in cancer associated with trisomy while studying patients diagnosed with AML and +11 as a sole cytogenetic abnormality (4, 5). The gene, located at 11q23, called *ALL1* (6, 7), *MLL* (8), *HRX* (9), *or HTRX* (10), was found to be rearranged in 10 of 11 (91%) AML patients with +11 as a sole cytogenetic abnormality, without cytogenetic abnormality at 11q23. In each case where additional material was available, the *ALL1* gene was found to have undergone a partial tandem duplication of an internal portion of itself (4, 5, 11). More recently, we have discovered that approximately 11% of adult patients with *de novo* AML and normal cytogenetics have rearranged *ALL1* as the result of a partial tandem duplication of the gene (12). More than 90% of the partial duplications span either exons 2 through 6 or exons 2 through 8, and each has been identified in adult *de novo* AML cases with +11 or normal cytogenetics. In all cases, the unique fusion resulting from partially duplicated sequence of the *ALL1* gene has been found to be in frame, predicting a partially duplicated protein product. The mechanism by which the partial tandem duplication contributes to leukemogenesis is currently unknown, as is the mechanism of all rearrangements involving *ALL1*. Moreover, the mechanism underlying the frequent specific association of +11 with *ALL1* gene mutation is unexplained.

In the current study, we analyze AML blasts from patients with the partial tandem duplication of *ALL1* and either +11 as a sole cytogenetic abnormality or normal cytogenetics. We demonstrate that in each instance, only one chromosome 11 contains the partially duplicated *ALL1* allele.

MATERIALS AND METHODS

Patients Specimens. Bone marrow or blood blasts were obtained from five patients who were diagnosed with AML and +11 as a sole cytogenetic abnormality (three patients) or AML and normal cytogenetics (two patients) (Table 1). The diagnosis of AML was based on standard French–American–British morphological and cytochemical criteria (13, 14). All five patients were previously shown to rearrange *ALL1* on Southern blot analysis with the B859 probe and were found to have the partial tandem duplication of *ALL1* by reverse transcription-coupled PCR and direct DNA sequencing (11, 12).

Cytogenetic Analyses. The criteria used to define a cytogenetic clone and descriptions of karyotypes followed the recommendations of the International System for Human Cytogenetic Nomenclature (15).

DNA Preparation and Probes for Southern Blot Analysis. Preparation of DNA and Southern blot analysis were carried out as described (11). The B859 probe was used in Southern blot analysis. B859 is an 859-bp *Bam*HI fragment of *ALL1* cDNA that spans the breakpoint cluster region defined by exons 5–11 of the *ALL1* gene (7).

Determination of Number of Chromosomes Carrying the Partial Tandem Duplication of *ALL1*. Cryopreserved bone marrow or blood specimens containing ≥90% leukemic blasts were utilized. In instances where specimens contained <90% leukemic cells (two cases), blasts were positively selected using a CD34+ biotin-avidin Ceprate column (Cellpro, Bothel, WA), according to the manufacturer's instructions; 95% blasts were recovered as determined by Wright–Giemsa staining. Genomic DNA was quantitated three times by UV spectro-

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0027-8424/97/943899-4$2.00/0
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Abbreviations: AML, acute myeloid leukemia; +, trisomy; UPN, unique patient number.

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Table 1. Allelic dosage of the partial tandem duplication of *ALL1* in AML blasts

UPN	Karyotype*	*ALL1* partial duplication*	Copies of *ALL1* exon 18†	Copies of *ALL1* exon 5†
C‡	46,XY	N/A	2	2
23	47,XX,+11	Exon 2–exon 6	2.92 ± 0.026	3.86 ± 0.071
56	47,XY,+11	Exon 4a–exon 6	2.99 ± 0.035	3.83 ± 0.075
108	47,XY,+11	Exon 2–exon 8	2.98 ± 0.024	3.89 ± 0.051
144	46,XX	Exon 2–exon 8	2.01 ± 0.023	3.05 ± 0.030
211	46,XX	Exon 2–exon 6	2.01 ± 0.003	3.13 ± 0.006

*As reported in ref. 11 for UPNs 23, 56, and 108, and ref. 12 for UPNs 144 and 211.
†As quantitated by phosphorimage analysis, relative to one-half of the signal intensity measured from the same amount of DNA obtained from normal diploid cells (C) hybridized to the same probe.
‡C, control of diploid cells from a normal donor; N/A, not applicable.

photometry and blotted (5 μg per well) onto a positively charged nylon membrane with a Bio-Dot SF Microfiltration Apparatus (Bio-Rad) for a slot blot hybridization procedure. A single-copy 2.2-kb genomic probe (2.2XX) from the *SCL* gene located on chromosome 1p was used to quantitate the amount of DNA loaded (16). A 528-bp cDNA probe from exon 18 of *ALL1,* derived from normal genomic DNA by PCR, was used as a single-copy probe for chromosome 11, as exon 18 lies outside of the duplication of *ALL1*. A 240-bp cDNA probe from exon 5 of *ALL1* was derived from normal genomic DNA by PCR and was used to determine the number of chromosomes containing the partial tandem duplication of *ALL1,* as exon 5 lies within the duplicated region (11). The probes were labeled with [^{32}P]dCTP and hybridized to the slot blot by standard techniques (17). DNA was quantitated by measuring the signal intensity of the blot with a Molecular Dynamics PhosphorImager and the IMAGEQUANT computer program (Molecular Dynamics). The recorded signal intensity values of 2.2XX, exon 5, and exon 18 probes were first corrected for any differences in DNA loading, as measured by the 2.2XX probe signal intensity from normal DNA and patient DNA. The relative signal intensity from patient DNA (Y_r) was calculated as follows: $Y_r = Y/(X/2)$, where Y is the exon probe signal intensity recorded from patient DNA and X is the exon probe signal intensity recorded from normal diploid DNA. The relative signal intensity from normal DNA (X_r) was calculated as follows: $X_r = X/(X/2)$. X_r is therefore always equal to two. Thus, the Y_r and X_r are graphed relative to one-half of the measured probe signal intensity from normal diploid DNA. Each blot was probed three times, and the results from each experiment represent the signal intensity (mean ± SEM).

RESULTS AND DISCUSSION

Specimen Characteristics. Specimens from five patients were analyzed (Table 1). All patients had AML classified by the French–American–British system as FAB M1 or M2. AML blasts from unique patient numbers (UPNs) 23, 56, and 108 had +11 as the sole clonal cytogenetic abnormality; UPNs 144 and 211 had normal cytogenetics. All patients demonstrated *ALL1* gene rearrangements by Southern blotting, and the partial tandem duplication of *ALL1* by reverse transcription-coupled PCR and direct DNA sequencing. Each partial tandem duplication included exon 5 (11, 12).

Determination of Number of Chromosomes Carrying the Mutated *ALL1* Allele. When DNA obtained from diploid cells of a normal individual is hybridized, the signal intensity with the *ALL1* exon 18 probe and the *ALL1* exon 5 probe is indicative of one copy of each *ALL1* exon on each chromosome 11. Therefore, an arbitrary value of two is assigned to each signal. In AML blasts containing +11 as a sole cytogenetic abnormality, the signal intensity with the *ALL1* exon 18 probe should be three, relative to the signal intensity detected in DNA from normal cells. This is because the extra chromosome 11 should contain a single copy of *ALL1* exon 18, downstream of the partially duplicated region of *ALL1* (5, 11). However,

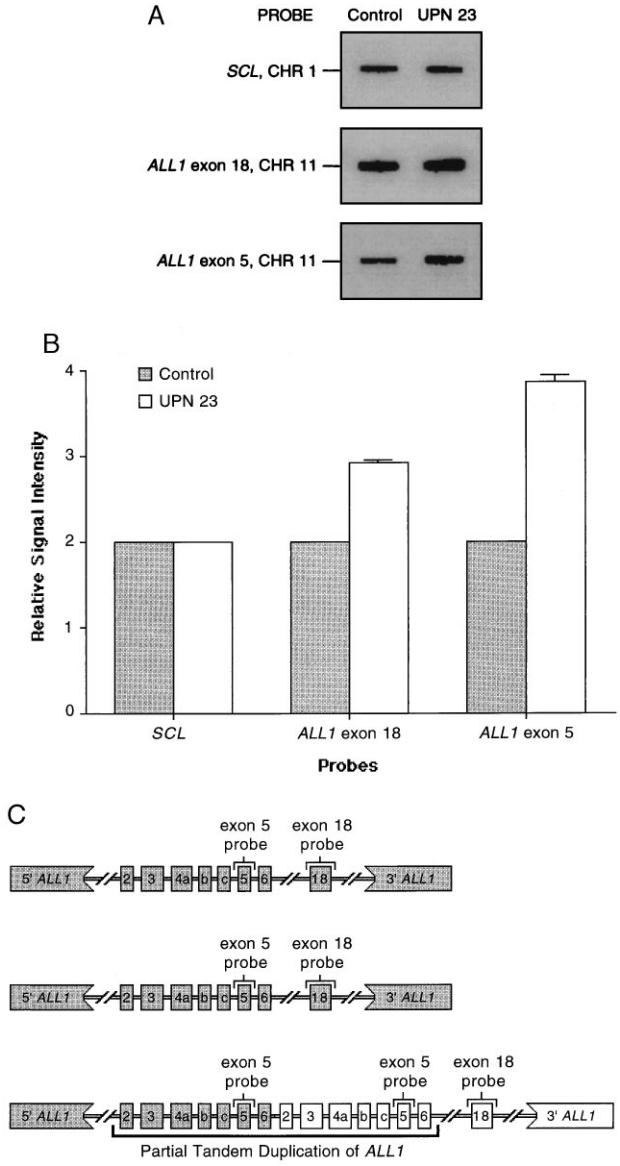

FIG. 1. Dosage of the wild-type and partially duplicated *ALL1* alleles in AML blasts of a patient (UPN 23) with +11 as a sole cytogenetic abnormality. (*A*) The pattern obtained with hybridization using *SCL*, *ALL1* exon 18, and *ALL1* exon 5 probes. The signal intensity detected with the *SCL* probe from chromosome 1 was the same for the normal control and UPN 23 (see Table 1), indicating that equivalent amounts of DNA were loaded. However, the signal intensity for the *ALL1* exon 18 and the *ALL1* exon 5 are visibly different when comparing the equal amounts of DNA from the normal control and UPN 23. (*B*) Graphical depiction of the quantitative phosphorimage analysis of signal intensity from *SCL*, *ALL1* exon 18, and *ALL1* exon 5 blots shown in *A*. Each measurement is relative to one-half the signal intensity in the normal donor using the same probe. This shows that UPN 23 with +11 had three copies of *ALL1* exon 18 that is outside the duplication, but four copies of *ALL1* exon 5 that is within the duplication. (*C*) A schematic diagram of *ALL1* allelic dosage in AML with +11 and the partial duplication, incorporating data obtained above. This shows that the three copies of *ALL1* exon 18 and four copies of *ALL1* exon 5 can be accounted for with only one of three chromosomes containing the partial tandem duplication of *ALL1.* The two remaining chromosomes 11 each contain a wild-type *ALL1* allele.

the relative signal intensity of the *ALL1* exon 5 probe could vary from four to six, depending on the number of chromosomes 11 carrying the mutated *ALL1* allele. Each chromosome 11 without the partial duplication of *ALL1* should have a relative signal intensity of one with the *ALL1* exon 5 probe, while each chromosome 11 with the partial duplication of *ALL1* should have a relative signal intensity of two with the *ALL1* exon 5 probe. AML blasts obtained from three different cases displaying +11 as a sole cytogenetic abnormality and the partial duplication of *ALL1* each had a relative signal intensity of three with the *ALL1* exon 18 probe and a relative signal intensity of four with the *ALL1* exon 5 probe. This is consistent with the AML blasts from each case having only one chromosome 11 with the partially duplicated *ALL1* allele (Table 1 and Fig. 1).

We quantitated the dose of wild-type and mutated *ALL1* alleles in AML blasts obtained from two patients with AML, normal cytogenetics, and the partial tandem duplication of the gene. The signal intensity measured with patient DNA hybridized to the *ALL1* exon 18 probe was equal to the signal intensity measured with an equivalent amount of normal donor DNA hybridized to the *ALL1* exon 18 probe (Table 1). This is consistent with the euploid number of chromosomes in both populations of cells and with the fact that the *ALL1* exon 18 is downstream of the partially duplicated region of *ALL1*. However, in both cases of AML, the signal intensity measured with patient DNA hybridized to the *ALL1* exon 5 probe was approximately 50% greater than the signal intensity measured with an equivalent amount of normal donor DNA hybridized to the *ALL1* exon 5 probe. This is consistent with three copies of *ALL1* exon 5 in the DNA obtained from AML blasts with normal cytogenetics, relative to two copies of *ALL1* exon 5 in normal cells (Table 1). Thus, in each of these two cases, only one chromosome 11 has a mutated *ALL1* allele containing two copies of *ALL1* exon 5 in the partial duplication of *ALL1*.

The partially duplicated *ALL1* allele contains both rearranged and germ-line DNA fragments on Southern blot analysis with the B859 probe (Fig. 2*C*). Hence, the presence of a germ-line band on analysis of leukemic blasts with the duplication does not in itself provide conclusive evidence for the existence of a wild-type *ALL1* allele. We therefore demonstrated the existence of a wild-type *ALL1* allele by assessing allelic dosage on Southern blot analysis with DNA obtained from a case of AML with the duplication spanning exons 2–6, normal cytogenetics, and 99% blasts. By using a *Hin*dIII restriction digest and the B859 probe, a germ-line band of 14 kb spanning exons 5–11 was noted with nearly double the intensity of the smaller rearranged band predicted by the partial duplication. No other bands were seen (Fig. 2*A*). A schematic of these data is shown in Fig. 2 *B* and *C*. These results demonstrate that in AML with normal cytogenetics and the duplication, one chromosome 11 has a wild-type *ALL1* allele. An identical Southern blot analysis was performed with >95% blasts from a case of AML with +11 as a sole cytogenetic abnormality and the duplication, demonstrating two wild-type *ALL1* alleles (data not shown).

In the current study, we establish that the partial tandem duplication of *ALL1* is found on a single chromosome in five cases of AML, regardless of whether the karyotype of AML blasts is normal or displays +11 as a sole cytogenetic abnormality. Thus, this work demonstrates that a single mutated *ALL1* allele with the partial tandem duplication is sufficient for *ALL1*-associated leukemogenesis. The lack of an additional mutated *ALL1* allele in cases of AML with +11 as a sole cytogenetic abnormality also leads us to speculate that the extra copy of chromosome 11 containing the wild-type allele provides a selective growth or survival advantage over normal cells and over leukemic cells with only one wild-type and mutated *ALL1* allele. Indeed, despite a strong association between a variety of human malignant phenotypes and certain trisomies, there is little understanding of how trisomy may lead to neoplasia. Gene amplification in somatic cells does occur frequently in certain neoplasias (18, 19) and presumably plays a role in some cancers consistently associated with trisomy. It is conceivable that increased gene dosage of other loci on chromosome 11 could also contribute to, or be responsible for, a selective growth or survival advantage afforded by the +11. However, the documented absence of an extra copy of chromosome 11 in leukemic blasts that have normal cytogenetics and the *ALL1* duplication (12) suggests that the additional chromosome is not critical for *ALL1*-associated leukemogenesis.

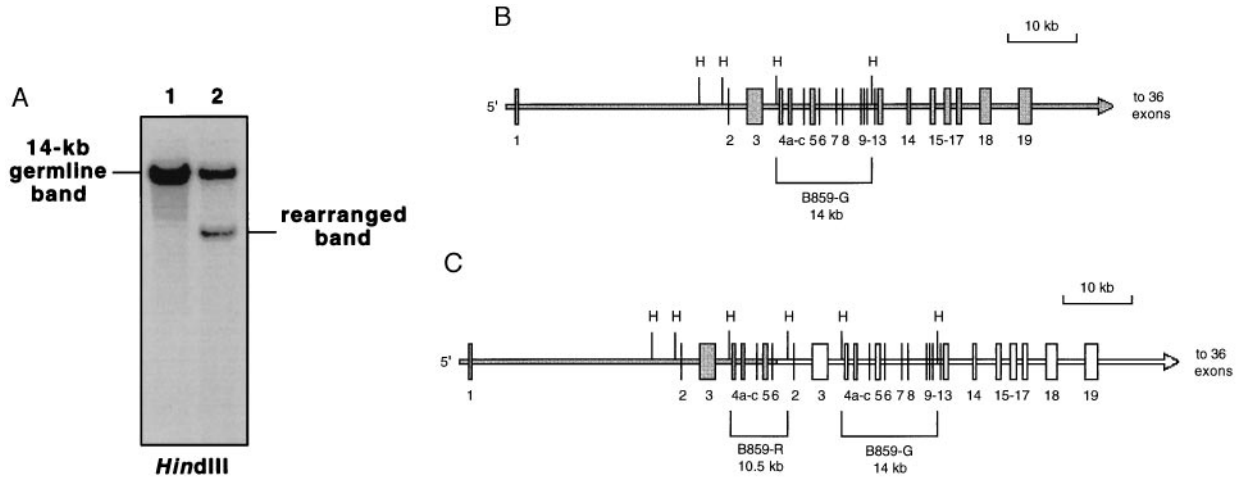

FIG. 2. Analysis of *ALL1* germ-line and rearranged bands in an AML patient with a normal karyotype (UPN 211) and the partial tandem duplication of *ALL1* spanning exons 2–6. (*A*) Southern blot analysis of *Hin*dIII digests of normal control (lane 1) and patient (lane 2) genomic DNA using the B859 probe. Patient genomic DNA was obtained from a specimen containing 99% blasts. Phosphorimage analysis showed the signal intensity of the patient's germ-line band to be approximately twice that of the rearranged bands (data not shown). (*B*) Schematic of the genomic structure of the wild-type *ALL1* allele showing location of *Hin*dIII (H) restriction sites within the gene. Numbered vertical lines and boxes indicate exons. The brackets indicate the 14-kb germ-line (G) *ALL1* band detected with the B859 probe after *Hin*dIII digestion. (*C*) Schematic of the genomic structure of the partial tandem duplication of *ALL1* involving a portion of the gene spanning exons 2–6, within the white region. The size of the rearranged (R) band that results after a restriction digest of genomic DNA with *Hin*dIII and hybridization with the B859 probe is indicated. Note that the chromosome 11 with the partially duplicated *ALL1* allele also has a germ-line band after *Hin*dIII digestion and hybridization with the B859 probe.

The mechanism by which the trisomy occurs in these cases also remains obscure. Cavenee *et al.* (20) postulated a chromosomal mechanism of mitotic nondisjunction with loss of the wild-type chromosome and reduplication of the mutant chromosome to account for a retinoblastoma containing three copies of a chromosome 13, each with evidence suggesting a mutant allele at the *Rb-1* locus. However, in striking contrast, the cases of AML with +11 that we studied appear to have two chromosomes 11 containing the wild-type allele at the *ALL1* locus and only one chromosome 11 with a mutant allele at the *ALL1* locus. This difference emphasizes the different modes of action of the *Rb-1* gene (a tumor suppressor gene whose mutations are recessive and lead to loss of function) and the *ALL1* gene (a likely transcription factor gene in which the tandem duplication may act dominantly through a gain of function). We postulate a mechanism of mitotic nondisjunction as the somatic genetic event to account for the +11 observed in some of our cases. We have evidence that *Alu*-mediated recombination is a molecular mechanism that can be responsible for *ALL1* partial tandem duplication observed in these somatic cells (ref. 21 and M.P.S. and M.A.C., unpublished observations). Whether the recombination occurs between two different chromosome 11 alleles or between sister chromatids of a single allele during mitosis is unknown. We cannot exclude the possibility that the molecular rearrangement and the emergence of trisomy occurred as different components of a single event. It is clear that in five cases of AML with the partial duplication and either normal cytogenetics or +11, the unaffected chromosomes 11 in the leukemic blasts have a wild-type *ALL1* allele.

In all 30 AML cases with the partial tandem duplication of *ALL1* and either normal cytogenetics or +11 studied thus far, RNA PCR and DNA sequence analysis have consistently revealed the unique fusion of exons to be in frame, demonstrating that the partially duplicated gene is transcribed into mRNA capable of encoding a partially duplicated protein (5, 11, 12, 21). Structural and functional evidence to date indicates that the wild-type *ALL1* is likely to encode a transcription factor that has a role in morphogenesis and hematopoiesis (22, 23). The manner by which the partially duplicated ALL1 protein may elicit leukemic growth remains uncertain. Dominant negative effects have been described in many such proteins and would be consistent with the dosage analysis of the mutated *ALL1* allele described in this report. Characterizaton of the wild-type and mutated ALL1 proteins is underway and should provide additional insights into its role in during normal hematopoiesis and leukemogenesis.

We thank Dr. Peter Aplan for his critical review of the manuscript and Ms. Dung Nguyen for her editorial assistance. This work was supported by Grants CA-37027, CA-65670, and CA-57974; a Translational Research Grant from the Leukemia Society of America; the Lady Tata Memorial Trust Fund; and the Coleman Leukemia Research Fund.

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