

# Lymphoma- and Leukemia-Associated Chromosomal Translocations in Healthy Individuals

Siegfried Janz,<sup>1\*</sup> Michael Potter,<sup>1</sup> and Charles S. Rabkin<sup>2</sup>

<sup>1</sup>Laboratory of Genetics, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, Maryland

<sup>2</sup>Division of Cancer Epidemiology and Genetics, National Cancer Institute, National Institutes of Health, Bethesda, Maryland

Chromosomal translocations (CTs) are hallmark mutations of hematopoietic malignancy that result in the deregulated expression of oncogenes or the generation of novel fusion genes. The polymerase chain reaction (PCR) can be used to detect illegitimate recombinations of genomic DNA sequences as a more sensitive assay than cytogenetics for determining the presence of CTs. Both direct DNA-PCR and reverse transcriptase-PCR were used to examine healthy individuals for lymphoma- and leukemia-associated CTs. Two oncogene-activating CTs [ $t(14;18)(q32;q21)$  and  $t(8;14)(q24;q32)$ ] and one fusion-gene CT [ $t(2;5)(p23;q35)$ ] from lymphomas and five fusion-gene CTs from leukemia [ $t(9;22)(q34;q11)$ ,  $t(4;11)(q21;q23)$ ,  $t(15;17)(q22;q11)$ ,  $t(12;21)(p13;q22)$ ,  $t(8;21)(q22;q22)$ ] were detected in such studies. The biological implication is that CTs associated with malignant tumors may also be found in cells that are not neoplastic. CTs are characteristic attributes of neoplastic clones but are by themselves insufficient to cause malignant transformation. A better understanding of the special biology of non-neoplastic CT-bearing cells will provide insight into their putative role as tumor precursors. Prospective epidemiological studies are needed to determine whether such cells in healthy individuals may, in some instances, become clonogenic founders of lymphoma or leukemia. © 2003 Wiley-Liss, Inc.

## INTRODUCTION

One of the triumphs of modern cytogenetics is the discovery of the tight associations of specific chromosomal translocations (CTs) with specific kinds of leukemias (Rowley, 1999), lymphomas (Kuppers and Dalla-Favera, 2001), and plasma cell tumors (Bergsagel and Kuehl, 2001). The predictable occurrences of the  $t(9;22)(q34;q11)$  in chronic myelogenous leukemia,  $t(14;18)(q32;q21)$  in follicular lymphoma, and  $t(8;14)(q24;q32)$  in Burkitt's lymphoma are some of the better known examples. These morpho-cytogenetic associations have led to the exciting discoveries of the genes juxtaposed at the chromosomal break sites and the emerging understanding of the biological effects of the rearrangements. The two main types of CT associated with lymphoma and leukemia are those involving oncogenes and those creating fusion genes. These studies have created an assumption that such CTs indicate the presence of hematopoietic malignancy.

In recent years, molecular methods such as polymerase chain reaction (PCR) have used the DNA sequence of the underlying illegitimate genetic recombinations to detect CT with greater sensitivity than that achieved by cytogenetics. Numerous studies of lymphomas and leukemias have demonstrated faithful correspondence between cytogenetic and molecular data in tumor analyses. The voluminous empirical data generated by these

studies seem to take for granted the equivalence of sequence-based and cytogenetic evidence of CT.

The PCR methods developed for tumor analyses have been extended to studying normal individuals for the presence of CT. The same CTs found in lymphoma and leukemia have been detected in healthy individuals. The first such study was reported in 1991 by Limpens and colleagues (1991) on  $t(14;18)(q32;q21)$ . Since this seminal publication, PCR evidence indicative of seven additional CTs in healthy human subjects has been obtained (Table 1). In addition, an experimental system in mice,  $T(12;15)$ , has been developed for a further understanding of oncogenic CTs in the absence of neoplasia.

The major difference between lymphoma- and leukemia-associated CTs is that the former primar-

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During the revision of the previously submitted (11/2001) first version of this commentary, a review article entitled "Leukemia- and lymphoma-associated genetic aberrations in healthy individuals" appeared in *Annals of Hematology* (J. Bäsecke et al., 81:64–75, 2002). The reader is referred to this interesting article for a more detailed discussion on  $t(9;22)$ ,  $t(14;18)$ , and  $t(2;5)$  in healthy individuals.

\*Correspondence to: Dr. Siegfried Janz, Laboratory of Genetics, NCI, Building 37, Room 2B10, Bethesda, MD 20892-4256.  
E-mail: sj4s@nih.gov

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TABLE I. Leukemia- and Lymphoma-Associated Chromosomal Translocation (LLA-CT) Reported in Healthy Individuals

LLA-CT	Neoplasia <sup>a</sup>	Percentage <sup>b</sup>	Gene/function <sup>c</sup>	Gene/function <sup>c</sup>	Precursor	Pathologic effect of CT <sup>d</sup>
t(14;18)(q32;q21)	FL	70–95	<i>IGH</i> at 14q32 Enhancers	<i>BCL2</i> at 18q21 Anti-apoptotic factor	Peripheral B-cell	Deregulated expression of <i>BCL2</i> by <i>IGH</i> enhancers (Knutsen, 1998; Bordeleau et al., 2000)
t(8;14)(q24;q32)	BL	85	<i>MYC</i> at 8q24 Transcription factor	<i>IGH</i> at 14q32 Enhancers	Peripheral B-cell	Deregulated expression of <i>MYC</i> by <i>IGH</i> enhancers (Boxer et al., 2001; Eisenman, 2001)
t(9;22)(q34;q11)	CML	90–95	<i>ABL</i> at 9q34 Tyrosine kinase	<i>BCR</i> at 22q11 Serin/threonin kinase	Hematopoietic stem cell	<i>BCR-ABL</i> fusion proteins Tyrosine kinase (Elliott et al., 2001; Laurent et al., 2001; Wong et al., 2001)
t(12;21)(p13;q22)	B-ALL in children	16–30	<i>TEL</i> at 12p13 Transcription factor	<i>AML1</i> at 21q22 Transcription factor	Precursor B lymphoblast	<i>TEL-AML1</i> fusion proteins Novel transcription factor (Loh et al., 1998; O'Connor et al., 1998; Rubnitz et al., 1999; Hiebert et al., 2001; Wiemels et al., 2001)
t(8;21)(q22;q22)	AML (M2)	20–40	<i>ETO</i> at 8q22 Transcription factor	<i>AML1</i> at 21q22 Core binding factor $\alpha$	Myeloid stem cell	<i>AML1-ETO</i> fusion proteins Novel transcription factor (Licht, 2001)
t(4;11)(q21;q23)	B-ALL	5–10	<i>AF4</i> at 4q21 Transcriptional activator	<i>MLL</i> at 11q23 DNA binding protein	Early precursor B lymphoblast	<i>MLL-AF4</i> fusion proteins Novel transcription factor (Li et al., 1998; Dimartino et al., 1999; Ayton et al., 2001)
t(2;5)(p23;q35)	ALCL	50	<i>ALK</i> at 2p23 Anaplastic lymphoma kinase	<i>NPM</i> at 5q35 Nucleophosmin	Activated mature cytotoxic T cell	<i>NPM-ALK</i> fusion proteins Novel tyrosine kinase (Duyster et al., 2001)
t(15;17)(q22;q21)	AML (M3)	>95	<i>PML</i> at 15q22 Nuclear regulatory factor	<i>RAR<math>\alpha</math></i> at 17q21 Retinoic acid receptor	Myeloid stem cell	<i>PML-RAR<math>\alpha</math></i> fusion proteins Blocks myeloid differentiation (Salomoni et al., 2002)

<sup>a</sup>Neoplasia in which CT commonly occurs: FL, follicular B-cell lymphoma; BL, Burkitt lymphoma; CML, chronic myelogenous leukemia; ALL, acute lymphoblastic leukemia; ALCL, anaplastic large cell lymphoma; AML, acute myelogenous leukemia.

<sup>b</sup>Prevalence of CT in indicated neoplasm.

<sup>c</sup>Rearranged gene and its physiologic function.

<sup>d</sup>See indicated reviews or primary papers for detailed information.

ily involve deregulation of existing oncogenes by the juxtaposition of immunoglobulin promoter/enhancer elements, whereas the latter generally generate fusion-genes encoding novel chimeric proteins [although t(2;5)(p23;q35) is a notable exception to this generalization]. Unlike oncogene-activating CTs, gene-fusion CTs tend to be spread out in long introns, and the actual breakpoint sequences can rarely be identified. However, the novel transcripts can be detected by reverse transcriptase (RT)-PCR, providing indirect evidence of the underlying translocation.

Thus, there are fundamental technical differences in the PCR assays for these two types of CT: (1) direct DNA-PCR utilizes rearranged genomic DNA as template, whereas RT-PCR first transcribes spliced mRNA into cDNA for amplification; (2) break sites are closely clustered in oncogene-activating CT, whereas breakpoints are spread out in introns in fusion-gene CT; (3) direct DNA-PCR can provide an estimate of clonal size because there is only one CT sequence per cell, whereas the variable number of chimeric transcripts prevents clonal quantitation with RT-PCR; (4) sequencing of a DNA-PCR product provides breakpoint information as a clonotypic marker, whereas the RT-PCR product does not because of removal of the genomic junction site by post-transcriptional splicing; (5) direct DNA-PCR may be affected by artifact attributed to template contamination, primer misannealing, and "jumping" of DNA polymerase, whereas RT-PCR has these possible artifacts plus additional pitfalls ascribable to reverse transcriptase-related errors. These issues require careful consideration as well as implementation of quality standards in the evaluation of studies reporting the presence of CTs in healthy individuals.

Detection of rare CT sequences among a predominance of germline sequences requires the use of highly sensitive PCR methods, with the consequent possibilities for artifact. These methods must be fully optimized to generate reliable evidence for the presence of CTs in a few cells of normal tissues. There are a number of criteria for evaluating whether a recombination sequence detected by nested PCR represents an actual CT rather than an artifact. Basic requirements for good laboratory practices for PCR include that DNA and RNA extraction, cDNA synthesis, preparation of PCR "master mix," primer dilutions, and both first- and second-step PCRs are each carried out in separate physical locations, and that patient and normal samples are handled separately, to minimize

the potential for cross-contamination. Preliminary evidence for CT exists when second-stage amplification is confirmed with multiple replicates, necessitated by the extreme sensitivity of nested PCRs and potential for artifact, and is stronger when the reciprocal products of CT can be detected. Suggestive evidence is provided by sequencing of direct DNA-PCR products, which identifies a unique breakpoint in genomic DNA; sequencing of RT-PCR products, which at a minimum must demonstrate an open reading frame, provides weaker support because cDNA lacks clonotypic breakpoints. Additional support may be provided by techniques such as cell sorting, which associate the CT with an appropriate hematopoietic lineage or maturation stage. Presumptive evidence includes re-isolation of DNA from the original sample with matching clonotypic breakpoint sequence or confirmation of RT-PCR data by detecting a corresponding CT in genomic DNA. Further confidence may be provided by demonstrating that junction sequences are consistent in longitudinal studies of single individuals, and that these sequences are unique (clonotypic) and not shared with similar junctions present in other individuals or reference samples used as PCR controls (e.g., CT-harboring cell lines or tumor tissues). Definitive evidence is the identification of the clonotypic breakpoint sequence in a subsequent neoplasia from the same individual. Clearly, attaining this highest level of evidence requires prospective epidemiological studies.

The biological and clinical associations of CT in overt hematopoietic malignancy (reviewed in Look, 1997; Rabbitts, 1999; Rowley, 1999) are summarized in Table 1. This commentary reviews published studies regarding the presence and biological implications of lymphoma- and leukemia-associated CT (LLA-CT) in healthy individuals. We generally refer to these abnormalities as CT, recognizing that in most instances only PCR evidence has been obtained.

#### ONCOGENE-ACTIVATING CHROMOSOMAL TRANSLOCATIONS

The oncogene-activating LLA-CTs t(14;18) and t(8;14) are associated with follicular lymphoma and Burkitt's lymphoma, respectively. Nested DNA-PCR studies have provided strong evidence that both of these CTs are present at low frequencies in healthy individuals (Table 2).

TABLE 2. Oncogene-Activating LLA-CT Detected in Healthy Individuals

CT	Gene	Tissue	Positives (prevalence)	Comment	Authors
t(14;18)	IGH-BCL2	Lymphoid tissues	13/24 (54%)	First report ever on CT in normals	Limpens et al., 1991
		Lymphoid tissues	10/25 (40%)	—	Aster et al., 1992
		PBL	29/53 (55%)	Positive correlation with age	Liu et al., 1994
		Spleen autopsies	11/31 (35%)	Positive correlation with age	
		PBL	65/122 (53%)	Positive correlation with tobacco smoking	Bell et al., 1995
		B cells	6/9 (67%)	4/6 positives harbored 2–5 distinct CT fragments	Limpens et al., 1995
		PBL	67/125 (54%)	Frequency of up to $450 \times 10^{-6}$	Ji et al., 1995
		PBL	34/34 (100%)	Positive correlation with age	Fuscoe et al., 1996
		PBL	39/64 (61%)	Frequency of $0.8-96 \times 10^{-7}$	Cole et al., 1996
		PBL, B-cells	26/57 (46%)	Frequency of $\sim 1 \times 10^{-6}$	Dolken et al., 1996
		CD22 <sup>+</sup> B-cells	4/32 (13%)	Evidence for oligoclonality	Fuscoe et al., 1996
		PBL	5/15 (33%)	Cell fractionation using CD22, FISH	Poetsch et al., 1996
		PBL	95 assays	—	Liu et al., 1997
		CD19 <sup>+</sup> B-cells	19/230 (8.3%) 5/34 (11.9%)	Positive correlation with exposure to sunlight	Bentham et al., 1999
PBL	39/241 (16%)	Normal individuals	Paltiel et al., 2001		
		First-degree family members of lymphoma patients			
		Japanese individuals	Yasukawa et al., 2001		
		German individuals			
t(8;14)	MYC-IGH	PBL	39/75 (52%) 2/99 (2%)	12/114 (10.5%) HIV-positive infected homosexuals	Müller et al., 1995a

**t(14;18)(q32;q21)**

Limpens et al. (1991) first reported detection of t(14;18) in non-neoplastic tissues, by the use of nested DNA-PCR to amplify recombination sequences between  $J_H/D_H$  at 14q32 and the major breakpoint region of *BCL2*. DNA sequencing revealed that the CT junctions occurred in the same break-site regions as in follicular lymphoma. At the limit of detection (one cell with CT in  $10^5$  non-affected cells), over half of the individuals tested were PCR-positive. Table 2 lists 14 studies from different laboratories that have confirmed and extended this landmark observation. Of particular significance was the detection of t(14;18) in CD19<sup>+</sup> peripheral-blood B cells, which are much easier to obtain than tissue biopsies. Liu et al. (1994) found t(14;18) in 29 of 53 individuals (54.9%), at frequencies ranging from 0.8 to 32 per  $10^6$  cells. For one of the positive individuals, a second sample was obtained 5 months later and found to be identical upon DNA sequencing, in the first reported use of DNA-PCR to demonstrate the persistence of aberrant cell clones (assuming PCR contamination did not occur). Limpens et al. (1995) used peripheral-blood lymphocytes from blood bank donors to recover two to five distinct clonotypic t(14;18) fragments from four of nine donors at different times,

thus establishing that multiple aberrant clones can co-exist in the same individual. A subsequent study of 26 t(14;18)-positive healthy blood donors confirmed this finding by demonstrating that six donors were positive for more than one junction sequence (Dolken et al., 1996). One donor with four unique recombination sequences had all four clones consistently detectable in nine samples obtained over a 6-year period. These data indicate that multiple abnormal clones may persist in some individuals over long periods of time without apparent development of lymphoma.

Follicular lymphoma is the most common type of non-Hodgkin lymphoma (NHL) in the United States and Europe, but is relatively uncommon among Japanese (<10% of all NHLs). Accordingly, epidemiological studies have investigated variation in circulating levels of the follicular lymphoma-associated CT t(14;18) among different geographic and ethnic populations. Aster et al. (1992) examined hyperplastic lymph nodes and tonsils from Americans and Japanese and found t(14;18) in similar proportions in both sets of individuals: 5 of 15 (33%) American vs. 5 of 10 (50%) Japanese. However, a much larger study of 241 healthy Japanese individuals and 75 healthy German individuals showed t(14;18) in 16% of the Japanese sample and

52% of the German sample ( $P < 0.0001$  in  $\chi^2$  analysis). Thus, the relatively low incidence of follicular lymphoma in the Japanese population may be related to a low frequency of t(14;18) rearrangements (Yasukawa et al., 2001).

Other investigators have begun to explore the potential of using t(14;18) as a molecular indicator of exposure to DNA-damaging agents. Although far from being definitive, the currently available studies have already demonstrated that the prevalence of t(14;18) is positively correlated with age (Liu et al., 1994; Ji et al., 1995), heavy smoking (Bell et al., 1995), and, curiously, exposure to sunlight (Bentham et al., 1999). Whereas the former observations are consistent with the view that translocation prevalence may be linked to general somatic mutagenesis (aging and smoking are both associated with elevated mutation loads), the latter finding has been interpreted indirectly by invoking the immunomodulatory effects of sunlight. Sunlight appears to trigger the release by skin cells of IL-6, an important growth and survival factor for B cells, which may boost the detection of t(14;18) by increasing the size and number of t(14;18)<sup>+</sup> cell clones (Bentham et al., 1999). However, the epidemiological implications are uncertain because sunlight exposure is apparently not related to NHL risk (Hartge et al., 1996).

Multiple independent studies that used DNA-PCR have found t(14;18)s to be highly prevalent in the normal population. One study that used fluorescence in situ hybridization (FISH) analysis has confirmed the PCR data (Poetsch et al., 1996). However, no attempt has yet been made to detect the reciprocal t(14;18) junctions in healthy individuals. Considering the limitations of these data, the evidence for the occurrence of t(14;18) in normal individuals may be considered to be presumptive. One reason why t(14;18) in B cells may be detected so frequently is that this translocation deregulates expression of the anti-apoptotic protein, BCL-2, providing a survival advantage and increased likelihood of clonal expansion.

#### **t(8;14)(q24;q32)**

Serial samples of peripheral blood lymphocytes collected over 12 years from 99 HIV-negative and 114 HIV-positive homosexual men provided material for determining the presence of t(8;14) CT, by use of primers annealing to *MYC* and *IGH* switch regions ( $S\mu$  and  $S\alpha$ ) (Müller et al., 1995a). Genomic DNA was prepared in a different (remote) laboratory and then submitted for PCR analysis as coded samples. Twelve of the 114 (10.5%)

HIV<sup>+</sup> individuals and 2 of the 99 (2%) HIV<sup>-</sup> individuals were t(8;14)-positive; both reciprocal products of t(8;14) were detected and confirmed by DNA sequencing. Sequence data further revealed the presence of multiple clones in some samples as well as diversification of some clones by base-substitution mutations and small deletions/duplications in *MYC*. In four of the HIV<sup>+</sup> cases, the same patient-specific recombination sequences were found on two or more isolations over periods of 1 to 9 years. Twelve of the HIV<sup>+</sup> men subsequently developed a lymphoma. Only one of the 12 men with lymphoma had t(8;14) detected in pre-lymphoma circulating lymphocytes; moreover, his tumor was negative for t(8;14). Thus, no clonal relationships between the t(8;14) in peripheral-blood lymphocytes and lymphoma development could be established.

The strength of the study by Müller et al. (1995a) is that t(8;14)s were consistent in longitudinal studies of single individuals (i.e., they were repeatedly detected). Furthermore, t(8;14)-positive cell clones were clearly defined by unique, perfectly matched, reciprocal junction sequences in the genomic DNA. However, only two HIV-negative individuals had positive results, and this study has not been repeated independently. Therefore, the evidence for the occurrence of t(8;14) in the absence of HIV infection may be considered presumptive.

### **GENE-FUSION CHROMOSOMAL TRANSLOCATIONS**

To date, five leukemia-associated and one lymphoma-associated fusion-type CT have been reported to occur in healthy individuals (Table 3). The quality of this evidence is generally weak, in that it is based on the detection of the fusion-gene transcripts by nested RT-PCR and is often not followed by sequencing of the cDNA. Confirmatory data to identify reciprocal fusion transcripts and/or corresponding genomic DNA junction sequences have not been obtained, with the notable exception of retrospective studies of pediatric leukemia patients, as detailed below.

#### **t(9;22)(q34;q11)**

In 1995, Biernaux et al. (1995) developed a highly sensitive nested RT-PCR strategy for detecting small amounts of *BCR-ABL* transcripts in chronic myeloid leukemia (CML) patients in remission (minimal residual disease). This methodology was based on generating and amplifying cDNA from 10<sup>8</sup> peripheral-blood lymphocytes,

TABLE 3. Summary of Fusion-Type LLA-IR Detected by RT-PCR in Healthy Individuals

CT	Gene	Tissue	Positives (prevalence)	Comment	Authors
t(9;22)	<i>BCR-ABL</i>	PBL	1/44 (2%)	0–15 years of age	Biernaux et al., 1995
		PBL	22/73 (30%)	20–80 years of age	
t(12;21)	<i>TEL-AML1</i>	Cord blood	12/16 (75%)	40 replicates	Bose et al., 1998
		PBL	1/67 (1.5%)	Outpatients	Eguchi-Ishimae et al., 2001
		PBL	11/99 (11%)	<20 years of age	
		PBL	2/48 (4%)	>20 years of age	
t(8;21)	<i>AML1-ETO</i>	Cord blood	6/567 (1%)		Mori et al., 2002
		Cord blood	1/496 (rare)		Mori et al., 2002
t(4;11)	<i>MLL-AF4</i>	Fetal liver	5/13 (38%)		Uckun et al., 1998
		Fetal BM	4/16 (25%)		
		Neonatal BM	1/6 (17%)		
t(2;5)	<i>ALK-NPM</i>	PBL	14/29 (48%)		Trümper et al., 1998
t(15;17)	<i>PML-RAR<math>\alpha</math></i>	PBL Lymphomatous	2/3	600 replicates	Quina et al., 2001
		PBL Granulomatous	1/3	600 replicates	

with analytic sensitivity capable of detecting even a single *BCR-ABL*-producing cell. Biernaux et al. (1995) and others (Talpaž et al., 1994; Hochhaus et al., 1995; Chomel et al., 2001) found *BCR-ABL* mRNA in blood cells of CML patients in complete cytogenetic remission after bone marrow transplantation. In the course of these studies, Biernaux et al. (1995) included a sample of peripheral-blood lymphocytes from a normal individual as a control, and unexpectedly detected *BCR-ABL* mRNA. They therefore went on to test 73 samples from healthy adults, and found P210<sup>*BCR-ABL*</sup> transcripts in 22 of 73 samples (30%). In a separate study, Bose et al. (1998) found *BCR-ABL* transcripts in peripheral-blood lymphocytes of 12 of 16 (75%) healthy adults: eight were of the P190 type, one was of the P210 type, and three were positive for both. In subjects with detectable *BCR-ABL*, one to 18 of 40 replicate aliquots tested positive in individual cases.

The studies by Biernaux et al. (1995) and Bose et al. (1998) made no attempt to detect the reciprocal *ABL-BCR* transcripts or the corresponding junction sequences in the genomic DNA. Furthermore, no attempts were made to redetect the *BCR-ABL* transcripts in the same individual at a later time. The studies by Biernaux et al. and Bose et al. have been widely quoted, but remarkably not repeated, and should thus be considered preliminary.

#### t(12;21)(p13;q22)

Eguchi-Ishimae et al. (2001), in an in vitro study on the origin of the t(12;21), demonstrated induction of a *TEL/AML1* fusion gene in cell lines by apoptogenic stimuli. This finding led them to test peripheral blood lymphocytes from 147 outpatients who had no history of hematopoietic malignancy or

exposure to immunosuppressive agents, as well as 67 cord blood samples. Thirteen patients (8.8%) and one (1.5%) of the cord blood samples were positive by RT-PCR. Mori et al. (2002) similarly found *TEL/AML1* fusion transcripts in 6 of 567 cord blood samples. The *TEL* and *AML1* breakpoints are known to cluster in specific introns in leukemia (Bernard et al., 1996). This facilitated the confirmation of the breakpoint sequences in the positive samples from this study by direct DNA-PCR. In summary, the studies by Eguchi-Ishimae et al. (2001) and Mori et al. (2002) provide preliminary evidence for the low-frequency occurrence of t(12;21) in newborns.

#### t(8;21)(q22;q22)

Detection of *AML1-ETO* transcripts is of particular clinical value because it helps to establish a definitive diagnosis of *AML1-ETO* rearrangements in the absence of cytogenetic evidence for t(8;21) (Langabeer et al., 1997), and a rise in fusion transcript levels may herald incipient clinical relapse (Marcucci et al., 1998a; Tobal et al., 2000). Mori et al. (2002) found one *AML1-ETO* transcript in 496 cord blood samples, providing the only evidence to date for the occurrence of this CT in normal individuals. Additional studies with considerably higher sample numbers must be performed before the significance of t(8;21) in newborns can be fully evaluated.

#### t(4;11)(q21;q23)

In 1998, Uckun et al. (1998) noted that *MLL* gene rearrangements may be found in infant acute lymphoblastic leukemia (ALL) patients who lacked t(4;11). By use of nested RT-PCR, they detected *MLL-AF4* transcripts in peripheral-blood

lymphocytes from 9 of 17 infants and 17 of 127 older children with ALL. Turning next to normal subjects, they also reported finding *MLL-AF4* transcripts in 4 of 16 fetal bone marrows, 5 of 13 fetal livers, and 1 of 6 normal infant bone marrow preparations, although no molecular details were given. Two subsequent reports, one based on 103 cord blood samples (Trka et al., 1999) and a second with 60 cord blood and 8 fetal liver samples (Kim-Rouille et al., 1999), failed to identify *MLL-AF4* transcripts by RT-PCR in relatively large series of normals, and thus the findings of Hunger and Cleary (1998) remain unconfirmed.

#### **t(2;5)(p23;q35)**

Trümper et al. (1998) reported the occurrence of *NPM-ALK* fusion transcripts in peripheral blood lymphocytes of 14 of 29 (48%) healthy individuals at a sensitivity threshold of one t(2;5)-harboring cell in  $10^7$  normal cells. The RT-PCR-positive samples in this study were confirmed by hybridization with radioactively labeled breakpoint-specific oligonucleotide probes, demonstrating that the PCR product was indeed the *NPM-ALK* fusion fragment. Two other groups have reported finding *NPM-ALK* transcripts in benign skin inflammation (Beylot-Barry et al., 1998) and in reactive lymphoid hyperplasia (Maes et al., 2001), lending support to the preliminary evidence for the occurrence of t(2;5) in healthy individuals.

#### **t(15;17)(q22;q21)**

Quina et al. (2001) attempted to induce t(15;17) in hematopoietic cell lines that lacked this translocation by exposure to 10 Gy of  $^{60}\text{Co}$  irradiation, but found that the non-irradiated control cells had nearly as many *PML-RAR $\alpha$*  transcripts as did the irradiated cells. Their findings prompted a study of peripheral-blood lymphocytes from three normal volunteers, in which 50  $\mu\text{g}$  of total RNA from  $2.5 \times 10^7$  cells was reverse-transcribed and the cDNA amplified through nested PCR. Seven samples among 600 aliquots analyzed were positive for *PML/RAR $\alpha$*  transcripts. The validity of this finding, which has not yet been independently confirmed, remains uncertain.

#### **Fusion Genes in Healthy Children With Subsequent Leukemia**

Studies by Ford et al. (1993, 1998) and Gill Super et al. (1994) on monozygotic twins with concordant leukemias provided the first indication that CT-bearing leukemia cells can have a prenatal origin. Concordant leukemias arise in one twin and

spread to the other via the shared placental circulation, with a variable and sometimes lengthy interval between disease onset in the two twins. Wiemels et al. (1999b) described a pair of twins that presented with t(12;21)<sup>+</sup> ALL at the ages of 5 and 14 years; remarkably, both leukemic populations possessed the identical *TEL-AML1* rearrangement, as determined by DNA sequencing of the translocation break sites. Other studies on the prenatal origin of acute leukemia took advantage of clonotypic junction sequences in chromosomal DNA obtained from archived neonatal heel-prick spots (Guthrie cards) matched to children who later contracted leukemia. Well-documented “back-tracked” cases have been reported for *MLL-AF4* fusions (Gale et al., 1997), *TEL-AML1* fusions (Wiemels et al., 1999a; Maia et al., 2001), and just recently *AML1-ETO* fusions (Wiemels et al., 2002). Clonotypic *IGH* or *TCR* recombinations have also been used instead of translocation breakpoints to establish the prenatal origin of pre-leukemic clones (Fasching et al., 2000; Yagi et al., 2000; Taub et al., 2002). In contrast to the studies of gene-fusion CT detected in the general population, these studies have identified both reciprocal fusion transcripts and additionally detected the matching sequence junctions in the genomic DNA. These studies provide solid evidence that CTs associated with childhood and infant leukemia are required, but not sufficient, to cause cancer (Greaves, 1999).

#### **MOUSE MODELS OF LLA-CT**

Mouse models provide additional biological understanding of the existence and implications of CT in the absence of lymphoma or leukemia. All LLA-CTs detected in healthy individuals have been recapitulated by transgenic technology in mouse models (Table 4), with the minor exception that *MLL-AF4* has been modeled only by other MLL fusion proteins (reviewed in Ayton and Cleary, 2001). The transgenic models have been invaluable in establishing the role of CT in the etiology of hematopoietic neoplasia, including their collaboration with oncogenes and tumor-suppressor genes during tumor development (Adams et al., 1999; He et al., 1999), their dependency on autologous regulatory elements (Westervelt and Ley, 1999), the requirement for both products of translocation to recreate certain neoplastic phenotypes (He et al., 2001), and the genetic mechanisms of translocation (Vanasse et al., 1999; Difilippantonio et al., 2000; Gao et al., 2000). Excellent reviews are available of the models for t(14;18) and t(8;14) (Adams et al., 1999; Boxer and Dang, 2001), t(9;22)

TABLE 4. Mouse Models of CT Associated With Leukemia and Lymphoma in Humans

Gene <sup>a</sup>	Type <sup>b</sup>	Human LLA-CT	Promoter/enhancer	Tumor phenotype	Comment	References
BCL2	TG	t (14;18)	E $\mu$	B-cell lymphoma	Several similar models	McDonnell et al., 1989, 1991
MYC	TG	t (8;14)	E $\mu$	Pre-B lymphoma	Several similar models	Adams et al., 1985
MYC	TG		E $\lambda$	Burkitt's lymphoma	—	Kovalchuk et al., 2000c
MYC	BAC		IgH enhancers	B-cell lymphoma	—	Butzler et al., 1997
MYC	KI		IgH enhancers	B/plasma cell tumors	—	Palomo et al., 1999
Myc/IgH	ICT		Endogenous IgH	Not reported	T(12;15) in ES cells	Janz et al., unpublished
Myc/IgH	SCT		Endogenous IgH	Plasmacytoma	T(12;15)	Smith et al., 1995
BCR-ABL	RD	t (9;22)	Viral promoters	CML-like and other leukemias	13 independent studies	Reviewed by Potter and Wiener, 1992
BCR-ABL	TG		MT	Various leukemias	8 independent studies	Reviewed by Wong and Witte, 2001
BCR-ABL	iTG		Tet-inducible promoter	CML	B-ALL on other background	Wong and Witte, 2001
BCR-ABL	KI		Endogenous BCR	B-ALL	120 days latency	Wong and Witte, 2001
TEL-AML1	RD	t (12;21)	MSCV	No leukemia	—	Reviewed by Andreasson et al., 2001
TEL-AML1	TG		E $\mu$	No leukemia	Normal hematopoiesis	Andreasson et al., 2001
AML1-ETO	iTG	t (8;21)	Tet-inducible promoter	No leukemia	Normal hematopoiesis	Reviewed by Licht, 2001
AML1-ETO	KI		Endogenous AML1	No leukemia	Embryolethality <sup>c</sup>	“
AML1-ETO	iKI		Tet-inducible promoter	No leukemia	Leukemia inducible with ENU	Yuan et al., 2001
AML1-ETO	iKI		Endogenous AML1	No leukemia	Leukemia inducible with ENU	Higuchi et al., 2002
AML1-ETO	ICT		—	Not reported	—	Buchholz et al., 2000
MLL-AF4	—	t (4;11)	—	—	No model available	Reviewed by Ayton and Cleary, 2001
MLL-AF9	KI		Endogenous MLL	Leukemia	Related model of t(4;11)	Corral et al., 1996
MLL-AF9	ICT		—	Not reported	Related model of t(4;11)	Collins et al., 2000
MLL- $\beta$ -gal	KI		Endogenous MLL	Leukemia	Related model of t(4;11)	Dobson et al., 2000
NPM-ALK	RD	t (2;5)	MSV, MSCV	B lymphomas	—	Reviewed by Duyster et al., 2001
PML-RAR $\alpha$	TG	t (15;17)	hCG, MRP8	Leukemia	—	Reviewed by Pandolfi, 2001
RAR $\alpha$ -PML	TG		hCG	No leukemia <sup>d</sup>	Responsive to ATRA	Reviewed by Pollock et al., 2001

<sup>a</sup>Expressed gene.

<sup>b</sup>Type of model: BAC, single-copy transgene based on bacterial artificial chromosome randomly inserted in genome; ICT, induced chromosomal translocation using site-specific recombinases, such as Cre or FLP; iTG, inducible transgene utilizing tetracycline-responsive promoter; KI, single-copy knock-in gene targeted to the desired genetic locus; iKI, inducible or conditionally expressed knock-in gene (e.g., using tet-on promoters or loxP-bracketed transcriptional stop cassettes that can be removed by Cre-mediated recombination); RD, retroviral transduction; SCT, spontaneously occurring chromosomal translocation; TG, conventional multicopy minitransgene randomly inserted in genome.

<sup>c</sup>Fusion gene caused embryonic lethality due to absence of normal fetal liver-derived definitive hematopoiesis.

<sup>d</sup>Not leukemogenic on its own, but increased the frequency of acute promyelocytic leukemia (APL) in doubly transgenic mice also harboring PML-RAR $\alpha$  (Pollock et al., 1999).

(Wong and Witte, 2001), t(12;21) and t(8;21) (Licht, 2001), t(4;11) (Ayton and Cleary, 2001), t(2;5) (Duyster et al., 2001), and t(15;17) (Pandolfi, 2001).

A general limitation of transgenic models is that they bypass the acquisition of CTs that naturally occurs as a stochastic, spontaneous, and rare somatic mutation event in tumor precursor cells (Westervelt and Ley, 1999). The one mouse model without this limitation is T(12;15), which is found in genetically unmanipulated mice as the murine homolog of the *Myc*-activating t(8;14)(q24;q32) in humans. This CT occurs spontaneously in untreated mice and as a frequent mutation in pristane-induced plasmacytomas. T(12;15) is an exceptionally relevant model for LLA-CT in humans, in that it affects appropriate target B cells (i.e., the plasmacytoma precursors), presumably by use of the same molecular machinery producing CT in humans.

T(12;15) has been detected repeatedly by a wide range of PCR methodologies in various genetic backgrounds, ages, and treatment conditions (Müller et al., 1994, 1996, 1997a,b; Kovalchuk et al., 1997, 2000a,b, 2001). The ability to identify and follow changes in the reciprocal *IgH-Myc* breakpoint junction sequences has provided a powerful tool to study the biology of aberrant CT-harboring cell clones, which has led to a number of important findings. The T(12;15) translocation appears to be a dynamic process that begins with a reciprocal exchange between *Myc* and the far upstream region of *IgH*, progresses by aberrant isotype switching that approximates *Myc* to the 3'-C $\alpha$  enhancer (Kovalchuk et al., 1997, 2000a), and then undergoes further clonal diversification by microdeletions in the junction flanks (Kovalchuk et al., 2000b). It is now known that CT is an early, if not initiating, event in inflammation-induced peritoneal plasmacytomagenesis in the mouse, given that *IgH-Myc*-bearing cell clones may be detected long before tumors arise (Janz et al., 1993). Comparison of tumor precursor cells and frank tumors indicates that the junctional region shortens during plasmacytoma development (Müller et al., 1994), a notable distinction from human Burkitt's lymphoma (Müller et al., 1995b). The breakpoint sequence has also been a useful clonotypic marker in studies of the mobilization and trafficking of *IgH-Myc*-bearing clones in mice undergoing tumor induction (Müller et al., 1997a) and in vivo propagation of premalignant clones (Kovalchuk et al., 2002).

There is a striking difference in genetic susceptibility to plasmacytoma development, with BALB/c

mice highly susceptible and DBA/2N uniformly resistant. Nevertheless, DBA/2N mice have been shown to generate *IgH-Myc*-containing cell clones, albeit with low frequency and minimal clonal expansion. After exposure to pristane, two of 20 (10%) DBA/2N mice compared to 32 of 44 (73%) of BALB/c mice were *IgH-Myc* positive, and the clone size in DBA/2N was smaller by semi-quantitative competitive PCR (Müller et al., 1996, 1997a). In a second study that used a more sensitive PCR method, the findings on DBA/2N were extended to two additional plasmacytoma-resistant strains (C3H/HeJ and C57BL/6). The three plasmacytoma-resistant strains were confirmed to have a lower prevalence of aberrant clones, 33% overall, compared to 91% for BALB/c (Müller et al., 1996, 1997a).

Environmental factors interact with host susceptibility to determine the frequency and outcome of T(12;15). Exposure to cholera toxin has been demonstrated to increase the frequency of *IgH-Myc*-bearing clones by three- to fivefold in both BALB/c and DBA/2N mice (Roschke et al., 1997), although the consequences for plasmacytoma development have not been examined. Administration of indomethacin to BALB/c mice undergoing plasmacytoma induction has been shown to result in a striking inhibition of plasmacytomagenesis by Potter et al. (1985, 1997) and Potter and Kutkat (1999), but there was no corresponding reduction of *IgH-Myc* recombination frequency in one study (Potter et al., 1997). Although there are significant gaps in our current knowledge about environmental influences on T(12;15), available data indicate that the inability to undergo translocation does not underlie resistance to plasmacytoma. Thus, plasmacytoma resistance must be governed by factors determining the subsequent fate of CT-harboring cells, such as their capacity to undergo apoptosis, cytokine support for clonal expansion, and cooperating genetic and epigenetic changes.

#### BIOLOGICAL COMPLEXITIES OF LLA-CT DETECTION IN HEALTHY INDIVIDUALS

A circumstance related to low-frequency LLA-CT in healthy individuals is minimal residual disease, in which leukemia patients in remission can have persistent clones with LLA-CT for many years without hematologic or clinical relapse (for reviews, see Birnie et al., 1990; Faderl et al., 1999; Basecke et al., 2002). Cell-sorting experiments have determined that the fusion gene may be present in multiple hematopoietic cell types, indicating that non-leukemic multipotential stem cells may persist in the bone marrow of acute leukemia

patients in remission (Miyamoto et al., 2000). Notably, RT-PCR may be insufficiently sensitive to detect fusion-gene–harboring clones, given that several investigators have found evidence for CT by FISH in patients with persistently negative RT-PCR (Dewald et al., 1998; Chomel et al., 2001). Fusion-gene products may be absent in primary samples from leukemia patients in remission but detectable after culture in vitro (Talpaz et al., 1994). It is uncertain how to reconcile these findings because they may reflect in vitro activation of transcriptionally silent LLA-CT, or reflect selection/expansion of transcriptionally active sub-clones. Nevertheless, these considerations suggest that the prevalence of CT in healthy individuals may even be underestimated by studies relying only on RT-PCR.

On the other hand, an RT-PCR product is not infallible evidence of CT because artifacts may generate a positive signal in the absence of CT. Caldas et al. (1998) demonstrated that transcripts resembling partial tandem duplications of the *MLL* gene can be generated without genomic *MLL* rearrangements by exon scrambling. Leukemia cells are prone to a host of RNA processing abnormalities that can masquerade as CT (Goodman et al., 2001). Although trans-splicing of two different transcripts has not been specifically demonstrated, it is conceivable that chimeric transcripts in healthy individuals could be produced by this mechanism. Of similar concern is template switching (i.e., recombination of two emerging cDNA strands copied from two distinct RNA templates). Retroviruses can perform template switching during reverse transcription in vivo (Negroni and Buc, 2001a,b) and, apparently, retroviral reverse transcriptase is capable of template switching in vitro (Duke et al., 2001; Mader et al., 2001). Thus, trans-splicing and template switching need to be considered as potential sources of fusion-gene products in evaluating whether PCR evidence is indicative of an underlying CT.

Increased prevalence of LLA-CT could be related to a more generalized tendency to genomic instability. LLA-CT in healthy individuals may co-exist with a whole spectrum of other mutations that have not been elucidated. In studies to date, the frequency of t(14;18) in healthy individuals has not been correlated with two other presumed markers of abnormal V(D)J recombination, *HPRT* exon 2/3 deletions (Fusco et al., 1997), and TCR $\beta/\gamma$  locus inversions (Meydan et al., 1999). However, other oncogene mutations have also been detected at low frequency in healthy individuals, including *MLL* partial tandem duplication

(Marcucci et al., 1998b) and *RAS*-activating point mutations (Wilson et al., 2000). It is not known whether such mutations attributed to entirely unrelated mechanisms are correlated with measures of LLA-CT.

## CONCLUSIONS

Exclusive reliance on PCR is not adequate to address outstanding biological questions. Although working with rare cell types is technically challenging, it would be desirable to have methods for isolating LLA-CT–bearing cells from healthy individuals. Surface marker analyses, gene expression profiles, and co-existing genetic and epigenetic alterations could help to define the precise nature of these cells. Additional insights could be garnered with methods for determining their tissue distribution, trafficking, longevity, and microenvironmental influences, to name but a few potential areas for future studies.

LLA-CT may be silently present in healthy individuals if they are protected from the corresponding neoplasms by tumor resistance or modifier genes. Alternatively, neoplasia may or may not develop because of critical environmental factors. Perhaps some healthy individuals inadvertently suppress aberrant CT-harboring clones by pharmacologic or dietary influences (e.g., statin inhibitors, anti-inflammatory drugs, antioxidant vitamins, green tea, etc.). It may be that PCR-detected LLA-CT occurs in cells that are not competent for neoplastic progression because of inappropriate lineage or the wrong stage of differentiation. Furthermore, there is evidence that fusion genes can be the source of unique antigens, and that immune responses could potentially regulate the survival and persistence of cells harboring fusion genes (Pinnilla-Ibarz et al., 2000).

The clinical and epidemiological significance of PCR-detected LLA-CT is not yet understood. Thus far, it has not been shown that healthy individuals harboring CT are at increased risk for developing the associated tumor types. Epidemiological studies are needed for identifying risk factors for CTs, and for examining the associations of the various mutations within individuals, their associations with other cancer risk factors, and, most important, their direct association with risk of subsequent lymphoma and leukemia.

Detection of LLA-CT in healthy individuals challenges the paradigm that these genetic accidents are oncogenic per se. Clones of cells with CT may undergo apoptosis, persist, or even expand without final transformation to malignancy. These alternative outcomes of affected cells represent

possible reasons for the dissociation of CT from hematopoietic malignancy. Because CT is much more frequent than neoplasia, malignant transformation must necessarily be rare, if it occurs at all. The recognition of CT in healthy individuals has been an important discovery, but much remains to be learned about its biological significance. The next generation of studies may lead to an improved understanding of both normal biology and cancer.

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