***BCL2* translocation frequency rises with age in humans**

(somatic mutation/aging/PCR/non-Hodgkin lymphoma/*BCL2*)

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ABSTRACT The background frequency of t(14;18) (q32;q21) chromosomal translocations at the locus associated with B-cell leukemia/lymphoma-2 (*BCL2*) was determined from a survey of the peripheral blood lymphocytes (PBLs) of 53 living individuals and from tissues of 31 autopsies by using a nested PCR assay. The translocation was detected in 55% of PBLs and 35% of autopsied spleens with a frequency of between less than 1 to 853 translocations per million cells. Translocations copurified with B lymphocytes. The frequency of translocations significantly increased with age in PBLs and spleens, as does human risk for lymphoma. Average translocation frequency was more than 40 times greater in the spleen and 13 times greater in the peripheral blood in the oldest individuals (61 yr and older) compared with the youngest individuals (20 yr or younger). Particular t(14;18)-bearing clones persisted over a period of 5 months in two individuals. These findings demonstrate that clones harboring the oncogenic t(14;18) chromosomal translocation are commonly present in normal humans, that such clones are long-lived, and that they rise in frequency with age. A multistep model of lymphomagenesis involving t(14;18) translocation followed by antigen stimulation is proposed.

It has been thought that most human tumor clones arise from a single transformed cell and that tumor progression is a multistep process requiring multiple mutational hits at oncogenes and tumor-suppressor genes (1–6). Since multiple hits at oncogenic loci are thought to be required for tumorigenesis, one might expect oncogenic mutations to occur in humans who have not yet developed tumors, given sufficiently sensitive techniques (7). The incidence of most tumors rises strikingly with age (8), and if mutational hits at oncogenic loci are a prerequisite for tumorigenesis, one might then expect the frequency of somatic mutations at oncogenic loci to rise with age. Although such expectations have existed for many years and evidence has accumulated that rare mutational hits accumulate at nononcogenic loci with age (9, 10), an age-related increase in mutations at an oncogenic locus has not been shown.

Non-Hodgkin lymphoma (NHL) is a tumor of B-lymphocyte origin, whose incidence rises more than 40-fold with human age (11). As in many other hematological neoplasms (12–14), specific chromosomal translocations occur in the two types of NHL, follicular lymphoma and diffuse B-cell lymphoma (15). The t(14;18)(q32;q21) translocation occurs in 85% of follicular lymphomas and 20% of diffuse B-cell lymphomas (11). Such translocations juxtapose the *BCL2* (B-cell leukemia/lymphoma-2) locus on chromosome 18 with the immunoglobulin heavy chain joining (*J*) region gene on chromosome 14, resulting in dysregulated expression of the *BCL2* gene (16, 17). Dysregulated expression of this locus

prolongs the survival of B cells, by delaying programmed cell death (18, 19).

We have developed a nested PCR assay for quantitation of rare t(14;18) translocations to test the hypotheses that such oncogenic mutations may occur (i) in persons without lymphoid neoplasia and (ii) in an age-dependent fashion.

MATERIALS AND METHODS

Preparation of DNA. Fifty-three blood samples from live persons and tissues from 31 autopsies were collected at the Los Angeles County Hospital, CA. Blood samples were kept at 4°C no more than 72 hr prior to lymphocyte separation. PBLs were prepared as described by Boyum (20) and were frozen at –20°C until DNA extraction. Autopsy tissues were maintained at –80°C prior to DNA preparation and then minced with a razor blade, and DNA was extracted by standard methods (21). All autopsy DNA samples were tested for degradation by a control PCR amplification with primers PA₁ and P₄ (5'-AAG GTC TGA TCA TTC TGTTC) (Fig. 1); samples that yielded a PCR signal when amplified for 30 cycles were used. Two spleen DNA samples were found degraded by this test and were excluded.

To generate B cell-enriched and -depleted pools of PBLs from which DNA was extracted, three t(14;18)-positive PBL samples were incubated with magnetic beads bearing anti-CD19 antibody (Dynal, Great Neck, NY) at a concentration of approximately three beads per B cell at 4°C for 1 hr with gentle agitation.

Nested PCR Assay Developed for t(14;18) Translocations. We developed and validated a rapid nested PCR assay (22–24) for t(14;18) translocations that does not require Southern transfer or radioactivity as does the existing assay (25, 26). Two micrograms of DNA was amplified with outer primers PA₁ (AGTTATGGCCTATACACTATTTGT) and P₄ (ACCTGAGGAGACGGTGACCAGGGT) for 30 cycles, and 0.4 µl of this first 30-cycle PCR product was transferred into the nested PCR for another 30 cycles with inner primers PA₂ (TTGTGAGCAAAGGTGATCGT) and P₃ (CAGGGTCCCTTGGCCCCAG) (Fig. 1). Primers PA₁ and PA₂ are homologous to the 5' sequence of the major breakpoint region (MBR), a hotspot for breaks near the *BCL2* gene (16); P₃ and P₄ are homologous to the consensus sequence of *J*₁ through *J*₆ (26). PCR conditions were 94°C for 5 min for denaturation, followed by 94°C for 30 sec and 55°C for 30 sec. DNA sequencing was done with primer P₂ (GCCTGTTTCAACACACAGACCC).

Reconstruction Experiment. To test the sensitivity and reliability of the nested PCR assay, several tests were performed. For the reconstruction experiment, pure t(14;18) target molecules were generated by amplification with PA₁ and P₄ of DNA from lymph node tissue of a patient with NHL (Fig. 2). The number of product molecules was quantified

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Abbreviations: PBL, peripheral blood lymphocyte; V, variable region gene; D, diversity region gene; J, joining region gene; MBR, major breakpoint region; NHL, non-Hodgkin lymphoma.

FIG. 1. Map of the nested PCR method and primers used to detect t(14;18) translocations. Primers PA₁, PA₂, P₃, and P₄ were used in the nested PCR assays for the t(14;18). chrom., Chromosome.

spectrophotometrically and then serially diluted into 2 μ g of DNA from a fetal sample from which t(14;18) was never detected in more than 30 trials. PCR detection of a positive signal became stochastic at a concentration of about 1 single t(14;18) molecule in a background of 600,000 normal haploid genomes, consistent with findings (25, 26) by others—i.e., that single t(14;18) molecules can be detected in a background of several hundred thousand normal genomes or more.

Size and Sequence Polymorphism of the t(14;18) Junction Fragment. Different junction fragment sizes were found in 10 of the 11 spleen samples positive for translocations (Fig. 3), supporting the notion that these signals were not the result of a single event of product contamination or *in vitro* PCR artifact but rather exhibited the size variation expected of breaks generated by the variable-diversity-*J* [*V(D)J*] recombinase *in vivo*. PCR products were then purified (Centricon 100) and directly sequenced (Fig. 4) by using 3.2 pmol of primer P₂ (except for the sample from individual 29, for which P₃ was used) and 0.5 pmol of double-stranded DNA template with a 373A automated DNA sequencer (Applied Biosystems). All nine determined sequences contained an identifiable fragment from the *BCL2* locus, an *N*-segment, and one of the six *J* regions, characteristic of breaks mediated by the *V(D)J* recombinase (27) (Fig. 4). The heterogeneity of the DNA sequence at these junctions supports the notion that the products amplified are t(14;18) translocations generated *in vivo*, rather than a PCR artifact generated *in vitro*. Samples from individuals 14 and 31 yielded multiple sequences. We infer that those spleens contain multiple clones of t(14;18)-bearing cells. Multiple bands were consistently amplified from the spleen of individual 14 (Fig. 3, lane 4), supporting the notion that multiple independent t(14;18) events may occur in one individual.

Confirmation of Nested PCR Assay by PCR-Southern Assay for Translocations. We tested the agreement of the nested PCR t(14;18) assays with the previously described method (25, 26, 28), which employs PCR amplification followed by

FIG. 2. Reconstruction test. A known number of t(14;18) targets were serially diluted into 2 μ g of spleen DNA from individual 5 and amplified by the nested PCR method in quadruplicate. Lanes: 1–4, 100 molecules on average; 5–8, 10 molecules on average; 9–12, 1 molecule on average; 13–16, 0.1 molecule on average; m, markers.

FIG. 3. Junction polymorphism test. PCR signals of all positive spleens, as well as the positive control (lymph node DNA of an NHL patient), were electrophoresed through a 2.5% agarose gel. Lanes 1–11, individuals 9, 10, 11, 14, 15, 17, 19, 20, 28, 29, and 31, respectively; 12, positive control; m, size markers.

Southern detection on 20 spleen samples. Amplification of 2 μ g of DNA with outer primers PA₁ and P₄ was performed for 30 cycles as described above, electrophoresed through 2.5% agarose, blotted to a nylon membrane, and probed with a fragment of the MBR prepared by primer extension of PA₁ as described by Medori *et al.* (29). The PCR-Southern method confirmed the nested PCR method for t(14;18) detection for all positives and all negatives in 20 representative samples (Fig. 5). In the experiment illustrated, samples from individuals 11 and 15 were negative by both nested PCR and PCR-Southern assay, consistent with the stochastic detection from these samples when 2 μ g of DNA is used.

Estimation of Translocation Frequency. The concentration of the t(14;18) translocation in 2 μ g of sample DNA was estimated by using a “multiple tubes approach” (7, 30), and Poisson assumptions. Five replicates of 2 μ g of PBL DNA (approximately 300,000 lymphocytes) and 10 replicates of 2 μ g of autopsy tissue DNA were assayed. It was assumed that one or more t(14;18) molecules would be detected with an efficiency of nearly 100%—a simplification that is justified based on our and other reconstruction experiments (7, 24–26, 31). The number of the t(14;18) molecules per tube was derived by the Poisson equation by substituting for λ the fraction of tubes positive for t(14;18) unless all replicates

Individual	18	N	14
9	GAAATGCAGTGGTGC.....GAGATA.....GGCTACTGGGGCC		
10	ATGCAGTGGTGCTTA...AGGGTGGGAGGGTGGACG.....CTACTGGGGCCAA		
11	GCAGTGGTGCTTACG.....T.....TACTGGGGCCAA		
14	AGCCCTCCTCCGC.....ATCTCCTCCTTGA.....TATGGACGTCT		
15	CTTCAGGGTCTTCC.....ATAATCGGG.....CTACTGGGGCC		
19	GCTTAGGCTCCACC.....GCTTGC.....ACTGGTTGACCC		
20	AAATGCAGTGC...CAGTGGTGGTAGCTGCTACTCGAAC...TACTACTACTACTA		
28	CCCTTTGACCTTGTTC.....GGACCTTGGGGG.....ACTTTGACTA		
29	TTTCTCATGGCTGTCTT.....CTCGGGGAATA.....ACTGGTTGACCC		
30	TCAGGGTCTTCAGAAA.....CTTAGTGAGG.....GACTACT		

FIG. 4. DNA sequences determined from nested PCR amplifications from 10 spleen samples; 18 refers to chromosome 18 breakpoint sequence, 14 refers to chromosome 14 breakpoint sequence at J₁ through J₆; and N refers to *N*-segment sequence.

FIG. 5. Comparison of nested PCR assay of t(14;18) junctions with the PCR-Southern assay. Spleen DNAs from individuals 20 to 1 (Table 1) were amplified with the outer primers PA₁ and P₄ for 30 cycles in a 50- μ l reaction mixture (lanes 1–20). A 2- μ l aliquot was taken into nested PCR, which contained the inner primers PA₂ and P₄ (A), and 30 μ l was blotted for Southern analysis (B). Negative results of individuals 11 and 15 agreed with the stochastic occurrence of positives in 10 replicates (Table 1). m, size markers.

were positive, in which case those samples were diluted and retested.

Criteria for Classifying Individuals As Negative or Positive for Translocations. When none of our replicate amplifications were positive (i.e., zero translocations detected in >1 million cells), the t(14;18) frequency in that individual was scored as zero. When one or more amplifications were positive, the individual was scored as a positive. Our estimate of the false positive rate for scoring a translocation is less than 1 in 40 because none of 40 “no DNA” controls produced PCR

positives. The safeguards and guidelines for detection of rare mutations by PCR put forth in ref. 7 were strictly adhered to.

RESULTS AND DISCUSSION

Rare *BCL2* Translocations Occur in B Cells of Lymphoid Tissues of Persons Without Lymphoma. t(14;18) mutation frequency was surveyed in autopsies known not to have lymphoma or lymphoid hyperplasia. Spleen, lymph node, liver, cardiac muscle, brain cortex, and skeletal muscle samples were assayed for the translocation (Table 1). Spleen samples, a rich source of B lymphocytes, were most frequently positive for the translocation (11 of 31). All other tissues were negative for the translocation except the liver of individual 19; this same individual had the highest absolute translocation frequency of all spleen samples surveyed. The DNA sequence of t(14;18) junctions from spleen and liver of individual 19 was identical, indicating an isoclonal mutation origin. The number of tissue samples surveyed for lymph node, liver, cardiac muscle, brain, and skeletal muscle were 11, 11, 10, 6, and 10, respectively.

BCL2 mutations were also frequently detected in PBLs from living donors. Twenty nine of 53 individuals were positive for t(14;18) in 2 μ g of PBL DNA. An anti-CD19 antibody was used to construct B cell-enriched and -depleted pools from PBLs of three individuals. Amplification of 0.25 μ g of DNA in triplicate from each pool provided six positives in nine samples of the B cell-enriched pool and no positives in the B cell-depleted pool (data not shown), consistent with a B-cell origin of these positives. The high relative frequency of *BCL2* mutations in splenocytes and in PBLs is consistent with the model in which such mutations are the result of

Table 1. Translocations per million cells

Subject	Age/sex	Cause of death	Tissues					
			Sp	Li	LN	Co	He	M
1	0/M	Stillborn	0	0	0	—	0	0
2	0/M	Stillborn	0	—	—	—	—	—
3	0/M	Prematurity	0	—	0	—	—	—
4	0/M	Hydrocephalus	0	—	—	—	—	—
5	0/M	Prematurity	0	—	0	—	—	—
6	4/M	Trauma	0	0	0	0	0	0
7	21/M	Trauma	0	—	—	—	—	—
8	27/M	Trauma	0	—	—	—	—	—
9	30/M	Asthma	2.7	0	0	0	0	0
10	33/M	Cerebral edema	5	—	—	—	—	—
11	38/M	Cardiomyopathy	5	0	0	0	—	—
12	42/M	Acute pyelonephritis	0	—	0	—	0	—
13	43/M	Cocaine overdose	0	—	—	—	—	—
14	49/M	Lung cancer	2	0	—	—	—	0
15	49/M	Coronary disease	213.3	0	0	0	0	0
16	50/M	Asthma	0	—	—	—	—	—
17	61/M	Brain cancer	13.3	—	—	—	—	—
18	69/M	Liver cancer	0	0	—	—	0	0
19	78/M	Coronary disease	853.3	2.7	—	0	0	0
20	81/M	Liver cancer	6.7	—	—	—	—	—
21	0/F	Prematurity	0	—	—	—	—	—
22	0/F	Prematurity	0	—	—	—	—	—
23	0/F	Prematurity	0	—	—	—	—	—
24	10/F	Trauma	0	—	—	—	—	—
25	33/F	Squamous carcinoma	0	0	0	—	0	0
26	42/F	Trauma	0	0	0	0	0	0
27	43/F	Brain cancer	0	—	—	—	—	—
28	46/F	Hypoxia pneumonia	2.7	—	—	—	—	—
29	56/F	Cervical cancer	3.3	0	0	—	0	0
30	77/F	Coronary disease	0	—	—	—	—	—
31	85/F	Trauma	5	—	—	—	—	—

0, not detected; —, not determined; Sp, spleen; Li, liver; Co, cortex; LN, lymph node; He, heart muscle; M, skeletal muscle.

errors of the *V(D)J* recombinase, an enzyme whose expression is limited to lymphoid cells. This lymphoid-specific distribution of mutation we report is consistent with reports by others that low levels of t(14;18) occur in human tonsils with hyperplastic lymphoid tissue (30, 32). These data do not support the claim that *V(D)J* recombinase may be active in brain (33).

There is Extensive Quantitative Variation in *BCL2* Translocation Frequency among Human Spleens and PBLs. Variation in *BCL2* mutation frequency in spleens and PBLs was determined by the multiple tubes approach that is described in *Materials and Methods*. Thirty-one spleen samples were amplified in replicates of 10 trials. Of the 31 20 were never positive, 1 was positive in 4 trials, 2 were positive in 5 trials, 1 was positive in 6 trials, 3 were positive in 7 trials, and 4 were positive in 10 of 10 trials. Translocation frequency varied from 2 per million lymphocytes to 854 per million lymphocytes—a range of 420-fold (Table 1).

For PBL samples, two μ g of DNA of the 53 PBL samples were amplified in replicates of five trials each. In those five replicate amplifications, 24 samples of the 53 were never positive, 15 samples were positive once, 6 samples were positive twice, 3 samples were positive three times, 2 samples were positive four times, and 3 samples were positive five times. Translocation frequency varied from 0.8 per million lymphocytes to 32 per million lymphocytes—a range of 40-fold (Fig. 6).

Frequency of the t(14;18) Translocation in PBLs and Spleens Is Significantly Correlated with Human Age. Given the known correlation between NHL incidence and human age, the correlation of t(14;18) frequency in PBLs versus age of donor was performed (Figs. 6 and 7). The mean t(14;18) frequencies per million PBLs $\pm$ SEM in four age categories (category 1 = 0–20 yr; 2 = 21–40 yr; 3 = 41–60 yr; 4 = 61 yr and older) were, respectively, 0.29 ± 0.13 , 0.77 ± 0.29 , 1.43 ± 0.80 , and 3.93 ± 1.58 (Fig. 7). There was a 13-fold increase on average in t(14;18) frequency between age category 1 and age category 4. The apparent increase in t(14;18) frequency was statistically significant by Spearman's rank correlation test of age versus translocation frequency ($P = 0.0067$, $r_s = 0.37$) and by a calculation of 95% confidence interval of t(14;18) frequency in the age categories 1 (0.29 ± 0.26) and 4 (3.93 ± 3.04).

A correlation of t(14;18) frequency in autopsied spleens with age was also performed. The calculated t(14;18) frequencies per million cells $\pm$ SEM were 0 ± 0 , 2.1 ± 1.0 , 24.7 ± 21.6 , and 146.3 ± 144.2 in the four age categories (Fig. 7). When we make the conservative estimate that there is one t(14;18) translocation or less per 2 μ g of DNA on average in the 10 spleens from age category 1, then there is a more than

FIG. 6. t(14;18) frequency in 53 PBL DNA samples versus age of donor.

FIG. 7. (Top) Mean frequency of the t(14;18) translocation in spleens vs. age. (Middle) Mean frequency of the t(14;18) in PBLs vs. age. (Bottom) Incidence of NHL vs. age (34). Error bars represent standard error of the mean in each age category.

40-fold increase in *BCL2* mutation frequency on average between spleens of age categories 1 and 4. The correlation between translocation frequency and age of donor was statistically significant (Spearman's rank correlation test, $P = 0.0005$, $r_s = 0.59$). The incidence of t(14;18) positives was also significantly higher among spleens 30 yr or older compared with spleens 29 yr or younger ($P = 0.01$, Fisher's Exact Test).

t(14;18) Clones Persist in Human PBLs over a Period of 5 Months. Two individuals whose PBLs were positive for t(14;18) and three individuals who were negative were resurveyed for t(14;18) after several months. The two individuals positive for t(14;18) at t_0 were reassayed at t_1 (where $t_1 - t_0 = 5$ months) and were found to be positive again. The junction sequence amplified at t_0 and t_1 from the first individual was: TGGTATGAAGCCAG-GTCCGGAAGG-GAAAAAC-TACTACTACTACTAC, exhibiting sequence patterns of the *BCL2* major breakpoint region, the N segment, and the use of J_6 . The amplified sequences from the second positive individual were TGAAATGCAGTGGTGC-AAACACAGTGGC-TACTACTACTACGGTATGG at t_0 , and TGAAATGCAGTGGTGC-AAACACTGGGCC-TACTACTACTACGGTATGG at t_1 , identical except for one change in the N region between t_0 and t_1 (shown boldface and underlined), which is most likely the result of somatic mutation. The observation of nearly identical breakpoints in PBLs drawn 5 months apart supports the argument that once t(14;18) translocations occur, clones bearing that translocation

FIG. 8. A possible model of lymphomagenesis.

tion tend to persist. Thus, the age-related rise in translocation frequency is likely the result of an accumulation of long-lived $t(14;18)$ -bearing clones. The three other individuals that were negative for $t(14;18)$ at t_0 were also negative at t_1 (where $t_1 - t_0 = 1$ year), consistent with the notion that forward mutation rate to $t(14;18)$ is low or that antigenic stimulation of $t(14;18)$ -bearing clones is infrequent.

Data Summary. Our observations of $t(14;18)$ translocation frequency and distribution are surprising in four respects. First, we were able to detect rare somatic mutations at an oncogenic locus in people without lymphoma or lymphoid hyperplasia. Second, the frequency of such mutations was highly variable among individuals. Third, the frequency of those mutations rose substantially with human age, as does the incidence of NHL. Fourth, the rise in $t(14;18)$ frequency appears to be the result of expansion of persisting clones, consistent with the function of the *BCL2* gene product.

$t(14;18)$ Frequency May Be Relevant to Risk for Lymphoma among the Aged. Somatic mutations at oncogenic loci are usually considered critical and irreversible events in tumorigenesis (4). Thus, one may expect that such mutations occur at higher frequency in cells prone to tumor development. It is known that the incidence of most tumors rises with age (8). Mutations in blood cells, including those caused by aberrant *V(D)J* recombinase activity, have been used as biomarkers of cancer risk (35, 36), and many of these assays detect an increase in mutation frequency with age in blood cells (37, 38). Aged people have at least a 40-fold higher risk for NHL, and we detected an increase of $t(14;18)$ frequency in the aged (Fig. 7). But the incidence of *BCL2* mutations in spleens (35%) and PBLs (55%) is high compared with the lifetime risk for NHL (0.6%). One might rationalize these facts by postulating a multistep model of lymphomagenesis in which $t(14;18)$ -bearing B cells reach numerical prominence by antigenic stimulation and subsequent malignancy by additional oncogenic mutations (Fig. 8). These results appear homologous to the situation in transgenic mice that bear a dysregulated human *BCL2* transgene in the germ line, in which development of follicular lymphoma is also rare (18, 39).

Are Expanded $t(14;18)$ -Bearing Clones the Precursors of Follicular Lymphoma? The $t(14;18)$ translocation at *BCL2* occurs in 85% of follicular lymphomas. Data presented here support the view that mutations at the *BCL2* locus occur frequently in humans without cancer and may be a common prerequisite for lymphoma. Our data lead to the predictions that (i) such $t(14;18)$ -bearing B-cell clones may persist over years, (ii) such clones may accumulate other mutations in growth-dysregulatory or lymphomagenic loci with age, and (iii) human groups at excess risk for lymphoma may have elevated $t(14;18)$ frequency.

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