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Risk of follicular lymphoma associated with *BCL2* translocations in peripheral blood

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Abstract

Many adults have circulating lymphocytes with the *BCL2* gene translocation characteristic of follicular lymphoma. We therefore conducted a nested case-control study of incident lymphomas with peripheral blood obtained median 4.9 years prediagnosis from the Prostate, Lung, Colorectal and Ovarian Cancer Screening Trial. Overall, 13 of 26 lymphoma cases and 14 of 47 controls had *BCL2* major breakpoint region (MBR) translocations in prediagnosis blood (odds ratio [OR]=2.8). Nine cases had *BCL2*-MBR-positive tumors; eight of these nine had *BCL2*-MBR translocations in paired blood *versus* five of the 17 with *BCL2*-MBR-negative tumors (P=0.01). Comparing both tumor types to controls, blood *BCL2*-MBR translocations had a strong, statistically significant association with *BCL2*-MBR-positive tumors (OR=26), but not with *BCL2*-MBR-negative tumors (OR=0.9). All eight *BCL2*-MBR-positive tumors with prediagnosis *BCL2* translocations were clonally related to these circulating cells, based on similarity of recombination sequences. These data indicate that blood *BCL2*-MBR translocations represent lymphoma precursor clones with malignant potential.

INTRODUCTION

The t(14;18) (q32;q21) translocation is the hallmark mutation and considered to be the initiating lesion of follicular lymphoma, the second most common subtype of non-Hodgkin lymphoma (NHL) [1]. The genetic rearrangement leads to constitutive activation of the *BCL2* gene, encoding the anti-apoptotic signaling protein B-cell lymphoma 2, by juxtaposition to the enhancer of the immunoglobulin (Ig) heavy chain locus, *IGH@* [2]. For treatment and follow-up of patients with follicular lymphoma, polymerase-chain-reaction (PCR) detection of these *BCL2* translocations provides a useful molecular marker for the

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POTENTIAL CONFLICTS OF INTEREST

None.

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presence of tumor cells in blood and other tissue compartments. High copy numbers can be detected in peripheral blood at the time of diagnosis, decreasing numbers in the circulation parallels clinical response and increases during remission predict imminent relapse [3].

Using similar PCR assays, it has long been recognized that as many as half of all adults have circulating lymphocytes with *BCL2* translocations [4]. Prevalence estimates vary depending upon assay sensitivity and number of genome copies analyzed. The translocation frequencies are relatively low, in the range of 0.1-1 copy per 100,000 nucleated blood cells in healthy individuals as compared to 100-1000 copies per 100,000 in follicular lymphoma patients prior to treatment [5]. Prevalence of *BCL2* translocations in healthy individuals has been variably associated with conditions related to NHL risk, including increased age [6], white race [7], male sex [8], heavy smoking [9] and pesticide exposure [10]. In addition, recently published data from two large prospective cohort studies indicates that healthy individuals with high frequency t(14;18)-positivity have increased risk of follicular lymphoma [11]. Here we present prospective associations of the presence and frequency of *BCL2* translocations in peripheral blood with subsequent risk of follicular lymphoma among participants of the United States National Cancer Institute's Prostate, Lung, Colon and Ovary (PLCO) study. We also analyzed the translocation sequences to identify clonal relationships of t(14;18)-bearing cells in peripheral blood with tumor cells in malignant lymphoma.

MATERIALS AND METHODS

Study Design and Participants

We conducted a case-control study nested within the intervention arm of the PLCO Screening Trial. In brief, 77,446 volunteers aged 55 to 74 years were randomly selected for systematic cancer screening at ten United States centers, as previously described [12]. Baseline questionnaires and peripheral blood samples were collected at study enrollment between 1993 and 2001. Participants were followed for incident diagnoses of any cancer by annual mailed questionnaires and by periodic screening examinations for the four targeted malignancies. All reported cancers were verified by medical record review for pathologic confirmation. Written informed consent was obtained from each subject, and institutional review boards of the National Cancer Institute and the ten study centers approved the trial protocol.

Between January 1994 and February 2008, 73 cases of follicular lymphoma (ICD-O-3 M-9690–9698) were identified among screening trial participants. Prediagnosis peripheral blood buffy coat samples were available from study enrollment for 26 of these subjects and for 47 cancer-free controls, individually matched 2:1 to cases for age, race, sex, study center and year of enrollment (5 cases had only one available matching control). The most frequent reason for sample unavailability was withholding of consent for DNA testing. Formalin-fixed, paraffin-embedded tumor biopsies were retrieved from respective hospital pathology departments for the 26 case subjects.

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Laboratory Procedures

DNA was extracted from buffy coat samples with Promega ReliaPrep kits, following the manufacturer's protocol (Promega Corp., Madison, WI). Tumor tissue was manually microdissected and DNA extracted with Gentra Puregene Blood kits (Qiagen GmbH, Hilden, Germany) into a total eluate volume of 50 µl. Quantitative real-time PCR was performed with consensus primers for the t(14;18) major breakpoint region (MBR) translocation of *BCL2* (*BCL2*-MBR) and for the wild type *KRAS* reference gene (two copies per genome), as previously described [13]. Standard curves for both assays were established from dilutions of DNA from the t(14;18)-positive B-cell line Karpas 422. Blood assays for t(14;18) used five replicates of 1 µg DNA, equivalent to testing a total of 750,000 nucleated cells, with sensitivity to detect a single translocation-positive cell. Tumor assays for t(14;18) were performed in triplicate using 1 µl of a 1:10 dilution of the DNA extract in each well; to exclude incidental translocations of adjacent normal lymphatic tissue, detection in all three wells was required for a tumor to be considered MBR-positive. Specificity of the PCR assays was confirmed by isolation of amplification products using agarose gel electrophoresis followed by sequencing with the BigDye Direct Cycle Sequencing Kit (Life Technologies GmbH, Darmstadt, Germany). Recombination sequences were compared to the GenBank database (http://www.ncbi.nlm.nih.gov/genbank) using the Basic Local Alignment Search Tool, which identified unique *BCL2*/*IGH* recombinations for each MBR-positive sample. t(14;18) frequency was calculated as the total number of copies in all five replicates, divided by the number of tested cells (i.e., one-half the number of *KRAS* copies). All experiments included appropriate positive and negative controls and laboratory personnel were blinded to case-control status.

For patients with *BCL2*-MBR-negative tumors, DNA was tested for the presence of non-MBR *BCL2/IGH*-translocations involving alternative *BCL2* breakpoints in 3'MBR, intermediate cluster region (icr) and minor cluster region (mcr), using similar assays. For details, see Supplemental Table I.

Statistical Analysis

We used conditional logistic regression models to estimate matched odds ratios (mOR) and 95% confidence intervals (CI) comparing lymphoma cases to their controls. We also used unconditional logistic regression to estimate odds ratios (OR) and 95% CI adjusted for the matching variables and smoking, and to fit adjusted multinomial models that included both MBR-positive and -negative tumors. Likelihood ratio tests of model goodness of fit were used to determine significance of covariables. Fisher and Freeman-Halton exact tests were used to assess two-way associations. Statistical analyses were performed with Stata 10 software (StataCorp, College Station, TX, USA). All statistical tests were two-sided, and *P*-values less than 0.05 were considered significant.

RESULTS

Distributions of selected matching variables for the 26 follicular lymphoma cases and 47 controls are shown in Table I; all subjects were white. In matched analysis, lymphoma patients were nonsignificantly less likely than their controls to have ever smoked cigarettes

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(*P*=0.06). Based on the consensus PCR assay for *BCL2*-MBR translocations, nine (35%) of the 26 case tumors were MBR-positive. Tumor MBR positivity did not vary between cases in ever smokers compared to nonsmokers (*P*=0.70).

Fourteen (30%) of the 47 controls had detectable *BCL2*-MBR translocations in peripheral blood buffy coat samples. Control blood translocations were not associated with age, sex or cigarette smoking (*P*>0.70 for each comparison). Thirteen (50%) of the 26 lymphoma cases had detectable *BCL2*-MBR translocations in pre-diagnostic blood samples. By conditional logistic regression, the mOR of follicular lymphoma for blood *BCL2*-MBR translocations was increased but not statistically significant (mOR=2.2, 95% CI=0.8 to 5.8). The association was slightly stronger in an unconditional analysis controlled for smoking as well as for the matching variables age, sex, study center and year of enrollment (OR=2.8, 95% CI=0.9 to 8.2). Study center and year of enrollment did not contribute to fit of the unconditional model (*P*=0.99).

BCL2-MBR translocations were detectable in prediagnosis blood DNA for eight (89%) of the nine subjects with *BCL2*-MBR-positive tumors and five (29%) of the 17 with *BCL2*-MBR negative tumors (*P*=0.01). Among the 17 *BCL2*-MBR-negative tumors, we identified three with *BCL2/IGH* translocations involving alternative *BCL2* breakpoints (non-MBR1-3, Table II). In two of these three cases, t(14;18)-positive cells with alternative *BCL2* translocations were present in circulating blood samples obtained three or more years before the clinical lymphoma diagnosis. Masked control samples tested simultaneously for each of these alternative translocations were PCR-negative in all instances.

The association of blood *BCL2*-MBR translocations with subsequent lymphoma greatly differed between tumors that were or were not *BCL2*-MBR-positive. In an unconditional multinomial model comparing both tumor types to controls, blood *BCL2*-MBR translocations had a strong, statistically significant association with *BCL2*-MBR-positive tumors (OR=26, 95% CI=2.5 to 270), but not with MBR-negative tumors (OR=0.9, 95% CI=0.3 to 3.2), adjusted for age, sex and cigarette smoking. In a sub-analysis restricted to tumors occurring more than 4 years after blood draw, four of five (80%) patients with *BCL2*-MBR-positive tumors had *BCL2*-MBR translocations in their stored pre-diagnostic blood (OR=21, 95% CI=1.3 to 300).

Despite the markedly higher prevalence, the number of copies of *BCL2/IGH* per 100,000 circulating blood cells was comparable to control frequencies for most of the cases of follicular lymphoma (*P*=0.08). Two MBR-positive tumor cases had copy numbers exceeding 10% of circulating cells and one MBR-negative tumor case had 32 copies per 100,000 cells, but the circulating levels for the remaining nine lymphoma cases were less than 10 per 100,000 cells (Table III).

All ten of the MBR or alternative *BCL2/IGH*-positive tumors that had circulating t(14;18) translocations prediagnosis were clonally related to these circulating cells, based on identical (nine cases) or near-identical (one case) translocation sequences (Table II). The composition of these sequences was generally indistinguishable from that of the t(14;18)-positive controls

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with respect to *BCL2* and *IGH* breakpoints, *JH* gene utilization and number of inserted nontemplated nucleotides (Supplemental Table II).

DISCUSSION

We found *BCL2* translocations in peripheral blood associated with subsequent risk of specific lymphomas. There was a borderline two- to three-fold increased risk of follicular lymphoma overall and a highly significant relative risk of 26 for MBR-positive tumors only. Restricted to cases diagnosed more than 4 years after blood sampling, the increase remained greater than 20-fold and statistically significant. Based on the correspondence of translocation sequences in DNA isolated from blood and tumors, our data indicate that most *BCL2/IGH*-positive follicular lymphomas arise from t(14;18)-bearing clones that circulate in peripheral blood for many years prior to diagnosis. These results extend the findings by Roulland *et al.* of increased risk of follicular lymphoma overall for individuals with high frequency ($> 10^{-4}$) of circulating *BCL2/IGH*-positive cells [11]. While two of our cases had very high translocation frequencies comparable to leukemic phase follicular lymphoma, the majority had frequencies in the same range as *BCL2* translocation-positive healthy individuals. Furthermore, by analyzing the tumors, our study specifically associates circulating MBR translocations with risk of follicular lymphomas bearing this same abnormality. While prediagnostic blood translocations were also detected in some subjects with MBR-negative tumors, the risk of MBR-negative lymphomas was not increased overall.

BCL2-MBR-positive tumors usually account for half of follicular lymphomas in large case series [14]. The relatively low percentage in our study may have been biased by false negative assays, attributable to the small quantity and low quality of DNA recovered from archival diagnostic biopsies. Alternatively, random fluctuation could be a sufficient explanation, based on the limited number of cases in our series.

The recombination sequences of t(14;18) among the general population resemble those of follicular lymphoma [15]. These translocations are thought to originate during early B-cell development in the bone marrow from aberrant *V(D)J* recombination that does not abrogate further maturation [13]. A single translocation sequence predominates within each individual, reflecting monoclonal rather than polyclonal derivation [16]. Using these sequences as clonotypic markers, it has also been shown that the expanded B-cell clones persist over years [17] and may evolve into clinical lymphoma [11,18,19]. The similar frequencies that we found in healthy individuals and in follicular lymphoma patients up to eight years prior to diagnosis suggests that *BCL2* translocation represents a pre-malignant condition–with the long latent intervals evidence against an alternative explanation of occult lymphoma.

BCL2 translocations in peripheral blood have been localized to atypical IgM memory (IgD+/CD27+) B-cells, long-lived lymphocytes with immunophenotypic markers resembling follicular lymphoma [13]. Similar to the cells in lymphoma, blood cells with *BCL2* translocations exhibit high levels of *BCL2* and activation-induced cytidine deaminase expression [20] as well as asymmetric class switch recombination [16]. The translocations and monoclonal patterns of *IGH* rearrangement of these circulating cells are also

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reminiscent of the abnormal germinal center cells described as follicular lymphoma *in situ*, a histologic diagnosis with a low rate of progression to clinically significant follicular lymphoma [21]. Additional molecular alterations may be required to confer malignant transformation of t(14;18)-bearing B-cell clones. Apart from *BCL2* translocations, follicular lymphoma tumors have recurrent somatic mutations in multiple genes thought to contribute to malignant transformation [22,23]. In one reported case of follicular lymphoma with prediagnosis lymphocytes, some of the lymphoma-associated mutations were also detected in the prediagnosis lymphocytes and others were present only in the tumor [18]. Investigation of additional cases is needed to distinguish whether clonal evolution depends upon a certain sequence of events, as opposed to accumulation of mutations at random.

Our data indicate that circulating *BCL2* translocations in clinically normal individuals represent lymphoma precursor clones with malignant potential, rather than a lymphoma risk factor. Recognition of *BCL2* translocations as a precursor condition for follicular lymphoma should facilitate further research toward a deeper understanding of lymphomagenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Table I

Selected characteristics of follicular lymphoma case and control subjects

	Cases		Controls	
	No.	(%)	No.	(%)
No. of subjects	26		47	
Male sex	16	(62)	28	(60)
Age at diagnosis, years				
median	70		70	
Range	59–82		59–81	
Ever smokers	9	(35)	26	(55)
Tumor translocation, MBR-positive	9	(35)	—	
MBR-negative	17	(65)	—	
Interval from blood draw to diagnosis, years				
median	4.9		—	
Range	0.4–12		—	

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Table II

Alignment of *BCL2*/*IGH* translocation sequences from matched samples of follicular lymphoma (FL) tumor and blood

Case no.	DNA source	Tumor histology (ICD-O-3)	Interval from blood draw to diagnosis (yr)	Trans-location frequency per 100,000 cells	*BCL2*-MBR break-point*	Other *BCL2* breakpoint	Nontemplated N nucleotides†	N length (bp)	*JH* gene	*IGH* break-point‡	PCR fragment length (bp)
MBR1	tumor	FL, grade 1 (M-9695)	0.4		3056		TGGGGACTAC	10	6	2967	134
	blood			16,600	3056		TGGGGACTAC	10	6	2967	134
MBR2	tumor	FL, NOS (M-9690)	0.7		3049		TGAGTT	6	4	1904	138
	blood			0.9	3049		TGAGTT	6	4	1904	138
MBR3	tumor	FL, NOS (M-9690)	1.8		3114		CTATTTCGGTT	11	6	2962	199
	blood			0.2	3114		CTATTTCGGTT	11	6	2962	199
MBR4	tumor	FL, grade 3 (M-9698)	3.0		3136		AGATTCCGT	9	5	2370	205
	blood			8.4	3136		AGATTCCGT	9	5	2370	205
non-MBR1	tumor	FL, grade 2 (M-9691)	3.2			16250‖ (icr)	GCCGACGGGGGGGGT	15	6	2954	222
	blood			5.4		16250‖ (icr)	GCCGACGGGGGGGGT	15	6	2954	222
MBR5	tumor	FL, NOS (M-9690)	4.5		3110		AGTATCACGTATTATCCCGGGGCGGTTTAA-**D**-GAAA	34	6	2949	246
	blood			10,600	3110		AGTATCACGTATTATCCCGGGGCGGTTTAA-**D**-GAAA	34	6	2949	246
non-MBR2	tumor	FL, NOS (M-9690)	5.3			16248‖ (icr)	GAATCCCTTAAT	12	6	2948	195
	blood			positive§		16248‖ (icr)	GAATCCCTTAAT	12	6	2948	195
non-MBR3	tumor	FL, grade 2 (M-9691)	5.8			2988*	GCGAAGAAACACGAAACAGATCGTCGACCCCTCATCAGATAA	42	4	1920	360
	blood			1.4¶							
MBR6	tumor	FL, grade 3 (M-9698)	6.7		3113		ACCCTATGGGTCTCCAT	17	6	2965	200
	blood			0.2	3113		ACCCTATGGGTCTCCAT	17	6	2965	200
MBR7	tumor	FL, grade 3 (M-9698)	7.1		3053		CCTAAGTGGGCTATTTTTTTTGTCT	25	6	2947	166
	blood			nonde-tectable							
MBR8	tumor	FL, NOS (M-9690)	7.8		3158		TCTGTC	6	4	1922	227
	blood			1.0	3158		TCTGTC	6	4	1922	227
MBR9	tumor	FL, NOS (M-9690)	8.2		3114			0	5	2366	176
	blood			positive§	3110		GCCAC	5	5	2371	174

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[*] *BCL2* sequence based on GenBank accession number M14745.

[†] **D** - indicates additional insertion of diversity gene *IGHD3–3*.

[‡] *IGH* sequence based on GenBank accession number J00256.

[§] Sample not quantitated due to assay failure.

[‖] *BCL2* sequence based on GenBank accession number AF325194.

[¶] Sequence not determined due to assay failure.

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Table III

BCL2-MBR translocation copies per 100,000 circulating blood cells before diagnosis of follicular lymphoma and controls

Frequency*	Cases	Controls	
0	13	33	
0.1–0.9	6	13	
1–9	3	1	
10–99	1	0	
100–999	0	0	
1000–9999	0	0	
10,000+	2	0	*P*=0.08 for frequencies > 0 Fisher-Freeman-Halton exact test

*Omits one qualitative positive case not quantitated due to assay failure.