Detection of t(14;18) carrying cells in bone marrow and peripheral blood from patients affected by non-lymphoid diseases

O Rauzy, S Galoin, J-J Chale, D Adoue, J-L Albarede, G Delsol, T Al Saati

Abstract

***Aims/Background*—To assess the presence of bcl-2/J_H rearrangements in bone marrow and peripheral blood lymphocytes from patients affected by diseases other than malignant lymphomas. The t(14;18) (q32;q21) translocation, which juxtaposes the bcl-2 oncogene on chromosome 18 and the J_H segment of the immunoglobulin heavy chain (IgH) genes on chromosome 14, is found frequently in follicular lymphomas.**

***Methods*—A sensitive semi-nested polymerase chain reaction (PCR) was used to detect t(14;18) translocation in bone marrow aspirates and peripheral blood lymphocytes from 48 patients. In 137 additional individuals peripheral blood lymphocytes only were tested.**

***Results*—Cells carrying bcl-2/J_H rearrangements were detected in about a quarter of the bone marrow samples and half of the peripheral blood lymphocyte samples. In seven patients, t(14;18) positive cells were found in both the bone marrow and peripheral blood lymphocyte samples. The size of the PCR products and bcl-2/J_H DNA sequence analysis showed that the same t(14;18) carrying clone was present in the bone marrow and the corresponding peripheral blood lymphocyte samples in three of these seven patients. Some patients had more than one bcl-2/J_H rearrangement. There was no significant correlation between age and the translocation incidence. Cells carrying the t(14;18) translocation were present in peripheral blood lymphocyte samples with a similar incidence—between 47% and 52% in all age groups from 20 to 79 years. Patients older than 80 years had a lower (37%) but not significantly different incidence.**

***Conclusions*—These findings suggest that patients affected by non-lymphoid diseases may have several t(14;18) carrying cells and some of them undergo a clonal expansion. Whether individuals with t(14;18) positive cells are at a higher risk of lymphoid malignancies remains unanswered and further epidemiological studies are required.**

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Keywords: t(14;18) (q32;q21) translocation; bone marrow; non-lymphoid diseases; semi-nested polymerase chain reaction

Department of Pathology and Unité de Physiopathologie Cellulaire et Moléculaire (UPCM) ERS 1590-CNRS, CHU Purpan, Avenue de Grande Bretagne, 31300 Toulouse, France
O Rauzy
S Galoin
G Delsol
T Al Saati

Department of Geriatric Medicine, CHU-Purpan, 31300 Toulouse, France
O Rauzy
D Adoue
J-L Albarede

Department of Biostatistics, Hôtel-Dieu, 31000 Toulouse, France
J-J Chale

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Correspondence to:
Dr Al Saati.

The t(14;18) translocation is a recurrent chromosomal abnormality found mainly in follicular lymphomas.[1] The t(14;18) (q32;q21) translocation juxtaposes the bcl-2 oncogene on chromosome 18 and the J_H segment of the immunoglobulin heavy chain (IgH) genes on chromosome 14. Junctional sequences of bcl-2/J_H rearrangement are similar to those generated by the normal rearrangement of IgH genes in bone marrow, including the addition of some non-germline nucleotides (N segment) by terminal deoxynucleotidyl transferase (TdT) at the junction of the two genes.[2 3] These findings suggest that the translocation occurs in pre-B cells when they attempt their first IgH gene rearrangement in the bone marrow. Recently, accumulating data have indicated that the t(14;18) translocation alone is not sufficient to confer a malignant phenotype to the cell. Additional genetic events involving other proto-oncogenes[4 5] are necessary for the development of malignant cell proliferations. Indeed, the t(14;18) has been detected in hyperplastic lymphoid tissues,[6] and in splenic and peripheral blood lymphocytes from healthy individuals and from patients affected by diseases other than malignant lymphomas.[7–11] Moreover, some of these studies showed that the frequency of lymphocytes carrying the t(14;18) translocation increases with age[7 9] and with smoking.[12] However, to our knowledge, the detection of t(14;18) carrying cells in the bone marrow of patients without lymphoid malignancy has not yet been reported.

Herein, we report the results of the detection of t(14;18) carrying cells in bone marrow and/or peripheral blood by polymerase chain reaction (PCR) from 185 patients affected by diseases other than lymphoid malignancies. We correlate the presence of these t(14;18) positive lymphocytes with age and with some other epidemiological and variable factors.

Materials and methods

POPULATION STUDIED

Owing to ethical and practical constraints, only bone marrow and peripheral blood lymphocyte samples that were taken for clinical diagnostic purposes were retrieved. In each case, only the sample residues, after the cytological and/or biochemical investigation, were retrieved for the bcl-2/J_H rearrangement study. All patients with lymphoid malignancies were excluded systematically. The patients were divided into two groups. In group I (n = 48), a bone marrow aspirate was obtained (table 1) and in 39 of these patients both bone marrow and

Table 1 Bone marrow cytohistological findings and clinical diagnoses

No.	*Age (sex)*	*Bone marrow cytological aspect*	*Final clinical diagnosis*	*BM*	*PB*
1	38 (F)	Normal	Hyperglobulinaemic purpura	−	+
2	89 (M)	Myelodysplasia	Sideroblastic anaemia	−	+
3	16 (F)	Reactive	Tuberculosis	−	−
4	66 (F)	Myelodysplasia	Sideroblastic anaemia	−	+
5	87 (F)	Normal	Benign monoclonal gammapathy	−	−
6	77 (F)	Hypoplastic	Anaemia of unknown origin	−	−
7	68 (F)	Normal	Peripheral neutropaenia	−	+
8	75 (M)	Myelodysplasia	Sideroblastic anaemia	−	−
9	60 (M)	Reactive	AIDS/fever	−	−
10	10 (M)	Normal	Sarcoma without BM metastasis	−	−
11	67 (F)	Normal	Pyoderma gangrenosum	−	−
12	31 (F)	Normal	Pneumocystosis/AIDS	−	−
13	76 (M)	Normal	Haemolytic anaemia	−	−
14	83 (M)	Reactive/megakaryocyte hyperplasia	Thrombocythaemia of unknown origin	−	+
15	78 (F)	Normal	Inflammatory syndrome	−	−
16	62 (M)	Normal	Benign monoclonal gammapathy	+	+
17	88 (F)	Myelodysplasia	Sideroblastic anaemia	−*	+
18	81 (M)	Myelodysplasia	Sideroblastic anaemia	−	−
19	15 (M)	Metastasis from sarcoma	Sarcoma with BM metastasis	−	−
20	75 (M)	Normal	Idiopathic cryoglobulinaemia	−	−
21	69 (F)	Normal	Renal carcinoma	−	−
22	4 (M)	Metastasis from neuroblastoma	Neuroblastoma with BM metastasis	−	+
23	2 (M)	Normal	Neuroblastoma without BM metastasis	−	−
24	73 (M)	Normal	Breast carcinoma	+	+
25	73 (F)	Reactive/haemophagocytosis	Infectious syndrome	−	+
26	45 (F)	Normal	Idiopathic cryoglobulinaemia	+	+
27	88 (F)	Normal	Idiopathic thrombocytopaenic purpura	+	+
28	87 (F)	Myelodysplasia	Sideroblastic anaemia	−	−
29	70 (F)	Normal	Peripheral thrombocytopaenia	−	−
30	88 (F)	Vitamin deficiency	Nutritional anaemia	−	−
31	87 (F)	Iron deficiency	Nutritional anaemia	+	+
32	56 (M)	Normal	Idiopathic cryoglobulinaemia	+	+
33	63 (F)	Myeloproliferative syndrome	Agnogenic myeloid metaplasia	−	+
34	73 (F)	Hypoplastic	Central thrombocytopaenia	−	+
35	68 (F)	Normal	Idiopathic neutropaenia	−	+
36	76 (M)	Normal	Anaemia of unknown origin	+	−
37	22 (F)	Normal	Mental anorexia	−	−
38	68 (M)	Normal	Idiopathic monocytosis	−	+
39	7m (M)	Normal	Neuroblastoma without BM metastasis	−	−
40	80 (M)	Myelodysplasia	Sideroblastic anaemia	−	ND
41	84 (M)	Normal	Thrombocythaemia of unknown origin	−	ND
42	24 (M)	Normal	Reactive adenopathy	−	ND
43	55 (M)	Normal	Amylosis	+	ND
44	70 (M)	Hypoplastic	Central thrombocytopaenia	+	ND
45	64 (F)	Hypoplastic	Central neutropaenia of unknown origin	+	ND
46	51 (M)	Normal	Spinal column pain	−	ND
47	58 (M)	Normal	Peripheral thrombocytopaenia	+	ND
48	3 (F)	Normal	Sarcoma without BM metastasis	−	ND

Results from 48 cases (group I) included in this study for the analysis of the presence of t(14;18) carrying cells in peripheral blood (PB) and/or bone marrow (BM) samples (10^6 mononuclear cells were examined for each patient).
F, female patient; M, male patient; ND, not determined.
*0.5×10^6 cells only were analysed.

peripheral blood samples were analysed. The patients in group I (24 men and 24 women) were aged from 7 months to 89 years (median age, 68 years). Table 1 summarises the clinical diagnoses and the results of bone marrow cytohistological examination, namely: (1) eight patients presented with haematopoietic disorders including seven cases of myelodysplastic syndrome (age: 66–89 years) and one myeloproliferative disease (age: 63 years); (2) eight patients had non-lymphoid malignancies associated either with bone marrow metastasis (one neuroblastoma and one sarcoma; ages: 4 and 15 years, respectively) or without bone marrow involvement (two neuroblastomas, two sarcomas, one renal carcinoma, and one breast carcinoma; ages: 7 months, 2, 3, 10, 69, and 73 years, respectively); (3) two patients had nutritional anaemia (ages: 87 and 88 years); (4) four patients had reactive bone marrow (ages: 16, 60, 73, and 83 years), of which three were associated with infection; (5) four patients had hypoplastic bone marrow (ages: 64, 70, 73, and 77 years); (6) in the remaining 22 patients, the bone marrow samples were normal in a clinical context of infectious or other benign pathological conditions (ages: 22–88 years).

Group II consisted of 137 patients (68 men and 69 women) from whom only peripheral blood lymphocyte samples were available. All were affected by diseases other than lymphoid malignancies and were aged from 18 months to 100 years (median age, 70 years). These patients presented with miscellaneous pathologies that fell into three main categories: neoplastic diseases (20%), infectious diseases (20%), and other varied minimal illnesses (60%).

DNA EXTRACTION

DNA was isolated from mononuclear cells as described previously.[13] The OCI LY8 cell line, which is positive for t(14;18) in the major breakpoint region (MBR),[14] and the CEM cell line (ATCC CCL 119), which is negative for the translocation, were used as positive and negative controls, respectively. In addition, standard precautions were taken to guard against cross contamination of the amplified DNA[15] and, in each experiment, a blank control (sterile water) was included. PCR products were expected to range from ~ 110–360 bp for the t(14;18) MBR. Amplification of the housekeeping gene c-raf-1 (258 bp fragment) was used as a positive control for

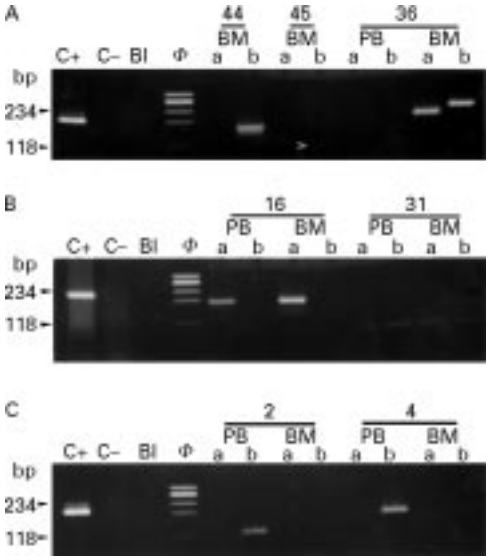

Figure 1 t(14;18) MBR translocation analysis by semi-nested PCR on bone marrow and peripheral blood lymphocyte samples obtained from group II patients. Two PCR runs (a and b) were performed for each sample using 0.5×10^6 mononuclear cells in each run. The amplified DNA was separated by 2% agarose gel electrophoresis and visualised by staining with ethidium bromide. C+ and C−, positive and negative controls, respectively; Bl (blank), PCR amplification without the DNA template to rule out contamination; Φ, size marker. (A) Patients 44 and 45 show a rearranged band in the bone marrow (BM) sample in only one PCR run. In patient 36, rearranged bcl2/J_H bands of different size are seen in the two PCR runs, indicating the presence of two different t(14;18) carrying cell clones in this bone marrow sample. (B) Patient 16 shows rearranged bands of the same size in only one of the two PCR runs performed for each bone marrow and peripheral blood lymphocyte (PB) sample. In patient 31, bcl-2/J_H bands of the same size are seen in the four PCR runs examined from the bone marrow and peripheral blood lymphocyte samples. (C) The two patients (2 and 4) show a rearranged band in the peripheral blood lymphocyte sample only in one of the two PCR runs performed.

successful amplification of the extracted DNA using the following primers: GAT GCA ATT CGA AGT CAC AGC G (Raf 8+) and TTT TCT CCT GGG TCC CAG ATA (Raf 9−).[16 17]

PCR CONDITIONS

Our study analysed the MBR of the t(14;18) translocation only,[18] using a semi-nested PCR method. The primary PCR reaction consisted of 50 µl of the mononuclear cell lysate (equivalent to 0.5×10^6 cells) and 50 µl of the following reaction mix: PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, and 0.001% gelatine), 200 µM dNTPs, 2.5 U AmpliTaq gold DNA polymerase (Perkin Elmer, New Jersey, USA), 50 pmol of the t(14;18) MBR specific primer (MBR3+, 5' TTT GAC CTT TAG AGA GTT GCT TTA CG 3') and 50 pmol of the consensus J_H region primer (JH−, 5' ACC TGA GGA GAC GGT GAC C 3').[17 19] The mixture was overlaid with mineral oil. For each sample, two PCR runs (a and b) of 35 cycles, using 0.5×10^6 cells/reaction, were carried out in a Perkin Elmer 480 DNA thermal cycler. The PCR cycle consisted of the following steps: denaturation at 94°C, annealing at 55°C, and extension at 72°C. The length of each step was one minute. An initial step of 10 minutes at 94°C to denature the DNA templates and to activate the AmpliTaq gold DNA polymerase was included, as was a final step of 10 minutes at 72°C to complete the reaction. The second PCR reaction consisted of 1 µl of the first PCR product and 49 µl of the same reaction mixture except for the addition of dimethylsulphoxide (DMSO) at a final concentration of 10%. In this second PCR, we used the primer JH− and an internal primer specific for the t(14;18) MBR (MBR-P+, 5' GCC TGT TTC AAC ACA GAC CCA C 3').[13 20] Thirty five cycles were performed; each cycle consisted of denaturation at 94°C for 45 seconds, annealing at 58°C for 35 seconds, and extension at 72°C for 35 seconds. Each experiment was repeated twice to exclude contamination as a source of error. Aliquots of 20 µl of each amplified product were applied to a 2% agarose gel electrophoresis and visualised by staining with ethidium bromide. The amplifications of the t(14;18) MBR showed rearranged bcl-2/J_H bands within the expected size range (110–360 bp).

Table 2 Sequences of bcl-2/J_H MBR junctions detected by semi-nested PCR

Patient	*Sample*	*bcl-2 MBR breakpoint*	*N region sequence/size (pb)*	*J_H breakpoint*
Group I				
2	PB-b	(3031)	tcctnccctccttccggccaagt (23)	J6 (2949)
4	PB-b	(3107)	cgcggattttggagtggttaac (22)	J6 (2949)
16	PB-a	(3113)	acccacggggtctggtactac (21)	J6 (2967)
31	PB-a,b	(3050)	ccctactggatagg (14)	J4 (1919)
	BM-a,b	(3050)		
32	BM-b	(3058)	gccaagt (7)	J6 (2949)
36	BM-a	(3110)	catataggcgggccaa (16)	J6 (2949)
	BM-b	(3170)	gggcggggg (9)	J4 (1925)
44	BM-b	(3113)	gggggatcg (9)	J6 (2963)
Group II				
1	PB-a,b	(3088)	gctgag (6)	J6 (2955)
2	PB-a	(3110)	(0)	J6 (2957)
	PB-b	(3052)	ttnggttgcatat (13)	J6 (2949)

Results of bone marrow (BM) and peripheral blood (PB) samples (group I) and peripheral blood samples only (group II). Two PCR runs (a and b) were performed from each sample, each equivalent to 0.5×10^6 mononuclear cells. Numbers in parenthesis indicate the nucleotide positions of bcl-2 and J_H breakpoints, based on the published sequences.

DIRECT SEQUENCING OF PCR PRODUCTS

To confirm the specificity of PCR products, we performed direct sequencing as reported previously.[13] Sequencing of the purified DNA was performed by the dideoxynucleotide chain termination method.[21] Both strands of the PCR product were sequenced with the primers used in the second round of amplification. The sequences obtained were compared with published germline sequences.[22 23]

SENSITIVITY OF PCR

As reported previously,[13] our bcl-2/J_H PCR technique could detect approximately one t(14;18) positive lymphoma cell diluted in 10^6 t(14;18) negative cells. Furthermore, we have shown recently, using single cell PCR methodology, that this technique can detect a single copy of a bcl-2/J_H sequence.[24]

STATISTICAL ANALYSIS

Correlation between t(14;18) translocation occurrence and ageing or smoking exposure in pack years was determined using analysis of variance. For the other epidemiological and variable factors (including sex, infection, neoplasia, chemotherapy, and radiotherapy) we

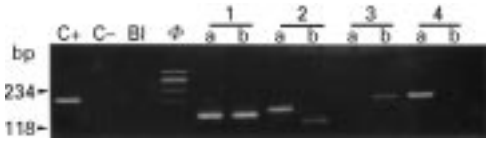

Figure 2 t(14;18) MBR translocation analysis by semi-nested PCR of peripheral blood samples obtained from group II patients (patients 1–4). Two PCR runs (a and b) were performed for each sample using 0.5×10^6 mononuclear cells in each run. The amplified DNA was separated by 2% agarose gel electrophoresis and visualised by staining with ethidium bromide. C+ and C−, positive and negative controls, respectively; Bl (blank), PCR amplification without DNA template to rule out contamination; Φ, size marker. Patients 1 and 2 show rearranged bands in the two PCR runs performed for each sample, of the same size or of different size, respectively. Patients 3 and 4 show only one rearranged bcl-2/J_H band each in one of the two PCR runs performed for each sample.

used the uncorrected χ^2 test. Probability values of less than 0.05 were considered significant.

Results

AMPLIFICATION OF THE BCL-2/J_H JUNCTION BY SEMI-NESTED PCR IN PATIENTS FROM GROUP I

As shown in table 1, bcl-2/J_H MBR rearrangement was detected in 11 of the 48 bone marrow samples analysed. For each sample, two PCR runs (a and b) were performed to test 1×10^6 cells. In four of the 11 bone marrow positive cases (patients 32, 36, 43, and 47), more than one type of bcl-2/J_H amplification product was seen, suggesting that these patients had several bcl-2/J_H carrying clones (fig 1A). In one additional patient (patient 31), the two separate PCR runs yielded bands of the same size, indicating a probable clonal expansion from the same bcl-2/J_H carrying cell (fig 1B). The rearrangements found in several patients were sequenced. As in follicular lymphoma, we found that most cases involved the J_H6 segment and a region N ranging from 7 to 23 bp (table 2). The DNA sequencing analysis of the bcl-2 junctional region in these cases confirmed the clonal relation (patient 31) or the presence of unrelated bcl-2/J_H bearing cell clones (patient 36) (table 2). In the six remaining cases, one band was detected in only one of the two PCR runs (fig 1A and B; table 2).

Peripheral blood samples were available for 39 of 48 patients and bcl-2/J_H gene rearrangement was detected in 18 of these. PCR runs showed one rearrangement in 14 patients (fig 1B and C) and different bands in four patients.

In 39 cases, results obtained from bone marrow and peripheral blood lymphocyte samples could be compared:

- In the seven patients with positive bone marrow samples, the corresponding peripheral blood lymphocyte samples were positive in six cases and, in three of these, the same clone was found in both samples (fig 1B). These findings were confirmed by DNA sequencing (table 2). In the remaining case, bcl-2/J_H positive cells were detected only in the bone marrow sample.
- In the 32 patients with negative bone marrow samples, the corresponding peripheral blood lymphocyte samples were either negative (n = 20) or positive (n = 12) (fig 1C). In three of the latter cases (patients 17, 22, and 25), more than one band was found, suggesting the presence of more than one t(14;18) bearing cell clone.

Statistical analysis did not show a significant increase in the t(14;18) detection rate with ageing, although in bone marrow samples the mean age was 69.6 years for t(14;18) positive patients *v* 58.4 years for t(14;18) negative patients. For these bone marrow samples, a significant correlation was found between cumulative smoking exposure in pack years (17.5 *v* 4 pack years) and occurrence of the translocation ($p < 0.014$). However, the comparison of patients with smoking habits and non-smokers was not significant ($p < 0.06$; $\chi^2 = 3.53$). Unexpectedly, there was an inverse correlation between infectious clinical status and the occurrence of the t(14;18) translocation ($p < 0.022$; $\chi^2 = 5.28$). No correlation was found between sex, neoplasia, chemotherapy, or radiotherapy and the t(14;18) detection rates.

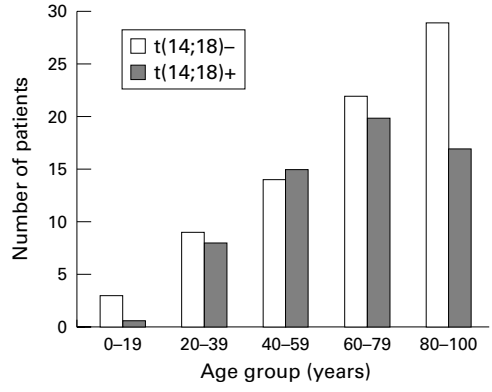

Figure 3 Distribution according to age groups of the t(14;18) positive and t(14;18) negative peripheral blood samples obtained from 137 patients affected by non-lymphoid diseases (group II).

AMPLIFICATION OF BCL-2/J_H JUNCTION BY SEMI-NESTED PCR IN PATIENTS FROM GROUP II

Sixty of the 137 (43.8%) patients were found to have cells carrying the t(14;18) MBR translocation. In 25 cases, one bcl-2/J_H rearranged band was detected (fig 2). In 13 patients, the same rearrangement was detected in the two PCR runs (fig 2). In the 22 remaining patients, more than one bcl-2/J_H rearranged band was found, suggesting the presence of more than one t(14;18) positive cell clone (fig 2). The latter hypothesis was confirmed by sequencing analysis in two cases (table 2).

As shown in fig 3, there was no correlation between the t(14;18) detection rate and ageing. Cells carrying the t(14;18) translocation were identified in peripheral blood lymphocyte samples at approximately the same incidence—between 47% and 52% in all age groups from 20 to 79 years. Patients older than 80 years had a lower (37%), but not significantly lower, detection rate. No correlation was found between sex, smoking habits, neoplasia, chemotherapy, radiotherapy, or infection and the incidence of the t(14;18) translocation.

Discussion

The results of our study confirm previous reports regarding the detection of t(14;18) positive lymphocytes in peripheral blood and they show, for the first time, that the latter cells can also be found in the bone marrow from individuals affected by non-lymphoid diseases. Overall, t(14;18) positive cells were detected in approximately half of the peripheral blood samples in healthy individuals or patients affected by non-lymphoid diseases.[7–11] Moreover, in several individuals, multiple bcl-2/J_H rearrangements were identified from the same bone marrow and/or peripheral blood lymphocyte samples. As reported previously by other groups,[6–11] these results suggest that multiple independent translocations occur within an individual. In addition, in three patients, the same bcl-2/J_H rearrangement was found in bone marrow and peripheral blood lymphocyte samples, suggesting that one t(14;18) positive cell had undergone clonal expansion.[6 7 10]

In contrast to the study by Ji *et al*,[9] and in agreement with another study,[10] we did not find any significant correlation between age and the translocation incidence. Of note, the relatively low rate of t(14;18) carrying cells in individuals aged more than 80 years was an unexpected finding. It could be attributed to the fact that those surviving above 80 years have lower genetic abnormalities. In our study, we did not estimate the frequency of t(14;18) positive cells. Such an estimation was performed by Liu *et al*,[7] who found that the mean t(14;18) frequencies for each million peripheral blood lymphocytes correlated significantly with age. In their study, there was a 13-fold increase on average in t(14;18) frequency between individuals aged from 1 to 20 years compared with those older than 61 years. These results have not been confirmed in two recent studies where the same approach was used.[11 12]

Recently, Bell and colleagues[12] have reported a significant correlation between smoking habits and the frequency of t(14;18) cells in the peripheral blood. In our study, we did not find any association between smoking exposure and the presence of t(14;18) positive cells in the peripheral blood. However, we found a significant association between cumulative smoking exposure and the occurrence of t(14;18) carrying cells in the bone marrow. Analysis of bcl-2/J_H gene rearrangement has suggested that the t(14;18) translocation occurs at an early stage of B cell development in the bone marrow.[2 3] The detection of t(14;18) positive cells in about a quarter of the bone marrow samples analysed in our study is in agreement with such a hypothesis. This suggests that precursor B cells carrying the t(14;18) translocation are generated regularly in the bone marrow of healthy individuals and that t(14;18) carrying daughter cells enter the blood and home to lymphoid tissues where they can be identified.[6–11]

Several studies have demonstrated the diagnostic value of PCR in detecting bone marrow involvement during and after treatment (that is, minimal residual disease) in follicular lymphoma associated with the t(14;18) translocation.[13 19 20 25 26] In this context, non-malignant t(14;18) positive cells are a potential source of false positive results. Consequently, the detection of a bcl-2/J_H rearrangement by PCR does not necessarily imply the presence of residual malignant cells in bone marrow. Whenever possible, the only means for differentiating these non-neoplastic t(14;18) carrying cells from the malignant t(14;18) positive cells is the use of clonospecific probes.[13] As reported previously, the sequence of these probes is based on the nucleotide sequence of the bcl-2/J_H junctional region generated by the t(14;18) translocation detected in the biopsy specimen taken at diagnosis.[13]

Our study shows that cells carrying a bcl-2/J_H gene rearrangement are detectable in bone marrow and/or peripheral blood lymphocytes from patients affected by diseases other than lymphoid malignancies. Whether individuals with t(14;18) positive cells are at higher risk of lymphoid malignancies is a question that requires further epidemiological studies. However, we know from t(14;18) transgenic mice overexpressing the bcl-2 protein that, in some animals, polyclonal follicular hyperplasia progresses over time to diffuse immunoblastic lymphoma.[4] It is postulated that the extended survival of the B cells in these mice increases the risk of additional mutations, a phenomenon that could also be involved in the development of human malignant lymphoma.[4]

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