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Clinical relevance of bcl-2(MBR)/J_H rearrangement detected by polymerase chain reaction in the peripheral blood of patients with follicular lymphoma

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Summary We evaluated the prognostic role of peripheral blood polymerase chain reaction (PCR) assay for detection of the bcl-2(MBR)/J_H rearrangement in 59 patients with follicular lymphoma (FL) treated at our centre since 1989. Thirty-five (59%) patients were bcl-2/J_H positive and 24 (41%) were negative in the peripheral blood at diagnosis. Peripheral blood bcl-2/J_H rearrangement detection at diagnosis had no relation to overall survival (OS) and time to progression (TTP). Peripheral blood PCR assay was performed post treatment in 17 patients who were bcl-2/J_H positive at diagnosis. Fourteen of the patients (82%, 95% CI 56–96%) became bcl-2/J_H negative. Nine of these patients were further analysed during follow-up and, after several months, circulating cells carrying the bcl-2/J_H rearrangement reappeared in five of the nine patients. Peripheral blood clearance of bcl-2/J_H-positive cells was correlated with better overall survival (log-rank $P < 0.05$) but not with TTP. Our data confirmed that bcl-2(MBR)/J_H rearrangement detection by PCR at diagnosis is not a prognostic factor in follicular lymphoma. In our series, clearance of circulating bcl-2/J_H-positive cells appeared to correlate with better overall survival. Post-treatment examination of the peripheral blood by PCR may have clinical relevance for prediction of the survival pattern of the patients.

Keywords: Bcl-2; t(14;18); polymerase chain reaction; follicular lymphoma; prognostic factors

The majority of follicular lymphomas (FL) and 20–30% of diffuse large B-cell lymphomas carry the t(14;18) (q32;q21) chromosomal translocation which juxtaposes the *bcl-2* gene on chromosome 18q21 to the joining region (J_H) of the immunoglobulin heavy chain gene on chromosome 14q32. This leads to overexpression of a functionally normal bcl-2 protein, conferring a growth advantage on the neoplastic cells (Yunis et al, 1987; Corbally et al, 1992; Croce, 1993; Mrozek & Bloomfield, 1993). The breakpoint regions on chromosome 18 are clustered in two areas, major breakpoint region (MBR) in 60% and minor cluster region (mcr) in 10–20% (Weiss et al, 1987; Cotter et al, 1990; Cotter et al, 1991) and are readily detectable by polymerase chain reaction (PCR) using the consensus J_H primers and primers specific to one or more breakpoint regions. This makes possible a sensitive and reproducible assay for detection of the bcl-2/J_H rearrangement at a level of 1 in 10^5 cells. The clinical significance of detecting the t(14;18) translocation in patients with FL remains controversial (Pezzella et al, 1992; Tilly et al, 1994; Johnson et al, 1995; Whang-Peng et al, 1995). Furthermore, the same rearrangement can be detected, although at very low levels, in healthy people, in whom it does not predispose to malignancy (Liu et al, 1994; Bell et al, 1995; Ji et al, 1995; Limpens et al, 1995; Dolken et al, 1996).

The peripheral blood is readily accessible and it has been suggested that it has a good degree of concordance with bone marrow (Hickish et al, 1991; Yuan et al, 1993) and with lymph node tissue (Lopez-Guillermo et al, 1996). We decided to evaluate prospectively the prognostic significance of bcl-2/J_H rearrangement detection by PCR in the blood of patients with FL treated at our centre.

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MATERIALS AND METHODS

Peripheral blood and/or bone marrow samples from fifty-nine HIV-negative patients with FL, histologically diagnosed by the International Working Formulation (IWF) (The Non-Hodgkin's Lymphoma Pathologic Classification Project, 1982) and the Revised European–American Classification of Lymphoid Neoplasms (Harris et al, 1994), were analysed in our department for the presence of the bcl-2(MBR)/J_H rearrangement using PCR assay during the period from January 1989 until December 1995.

All of the patients underwent a complete staging procedure, including physical examination, chest radiography, abdominal computerized tomography (CT) scan, routine haematology with bone marrow biopsy and aspiration and peripheral blood and bone marrow sample collection for assessment by PCR of the presence of the bcl-2(MBR)/J_H rearrangement.

Clinical stage was defined according to the Ann Arbor classification. Performance status (PS) was evaluated by the Eastern Cooperative Oncology Group (ECOG) scale. Bulky disease was defined as a mass > 10 cm. The International Prognostic Index (IPI) was calculated according to the original paper (The International Non-Hodgkin's Lymphoma Prognostic Factors Project, 1993).

Peripheral blood samples were collected during the initial evaluation in all patients and in 17 patients also after treatment.

DNA extraction

Whole-blood samples (10 ml) were treated with lysis buffer (0.32 M sucrose, 10 mM Tris-HCl pH 7.5, 5 mM magnesium

Table 1 Characteristics of the 59 patients with follicular lymphoma according to the detection by PCR of bcl-2(MBR)/J_H rearrangements in the peripheral blood

	Bcl-2/J_H-negative blood (24 patients)	Bcl-2/J_H-positive blood (35 patients)	*P* (χ^2-test)
Age (years)			NS
< 60	15 (63)[a]	23 (66)[a]	
> 60	9 (37)	12 (34)	
Sex			NS
Female	11 (46)	10 (29)	
Male	13 (54)	25 (71)	
Histology (according to the WF)			0.040
B	7 (29)	20 (57)	
C	15 (63)	15 (43)	
D	2 (8)	0	
Histological progression	1 (4)	2 (6)	NS
Ann Arbor stage			NS
I	7 (29)	6 (17)	
II	6 (25)	3 (9)	
III	4 (17)	9 (26)	
IV	7 (29)	17 (49)	
Systemic symptoms			NS
A	24 (100)	34 (97)	
B	0	1 (3)	
Performance status			NS
ECOG 0–1	24 (100)	32 (91)	
ECOG > 1	0	3 (9)	
Bulky disease			NS
Absent	22 (92)	32 (91)	
Present	2 (8)	3 (9)	
Serum lactate dehydrogenase			NS
Normal	18/23 (78)	28/32 (87)	
Elevated	5/23 (22)	4/32 (13)	
Unknown	1	3	
Serum β_2-microglobulin			NS
Normal	13/20 (65)	21/26 (81)	
Elevated	7/20 (35)	5/26 (19)	
Unknown	4	9	
Lymph nodes only	12 (50)	12 (35)	NS
Bone marrow involvement			NS
Absent	17 (71)	19 (54)	
Present	7 (29)	16 (46)	
Liver involvement			NS
Absent	23 (96)	32 (91)	
Present	1 (4)	3 (9)	
Spleen involvement			NS
Absent	21 (87)	28 (80)	
Present	3 (13)	7 (20)	
More than two extra-nodal sites	4 (17)	9 (25)	NS
International Prognostic Index			NS
Low risk	15/23 (65)	18/32 (56)	
Low–intermediate risk	6/23 (26)	9/32 (28)	
High–intermediate risk	2/23 (9)	4/32 (13)	
High risk	0	1/32 (3)	
Unknown	1	3	
Initial therapeutic approach			NS
Wait and see	9 (38)	13 (37)	
Chlorambucil with or without prednisone	7 (29)	12 (34)	
CVP	1 (4)	2 (6)	
CHOP-like	4 (17)	3 (9)	
Local treatment only (radiotherapy or surgery)	3 (12)	5 (14)	

[a]Numbers in parentheses are percentages.

chloride, 1% Triton X-100) to remove red cells. Pellets obtained by centrifuging (15 min at 3000 r.p.m.) were then incubated overnight at 37°C with 100 mM sodium chloride, 25 mM EDTA, 0.5% sodium dodecyl sulphate (SDS) and 200 μg ml^{-1} proteinase K (Sigma, St Louis, MO, USA). DNA was extracted by phenol–chloroform and precipitated with ethanol.

If small pellets were obtained after red cell lysis, they were resuspended in a digestion buffer [1 × PCR buffer, 0.5% Tween 20 and 200 μg ml^{-1} proteinase K (Sigma) for 100 μl] and incubated at 56°C for 90 min and then at 94°C for 20 min to inactivate enzymatic activity. DNA presence was tested with PCR assay using PCO4 (5′-CAACTTCATCCACGTTCACC-3′) and GH20 (5′-GAAGAGCCAAGGACAGGTAC-3′) primers (Perkin-Elmer, Norwalk, CT, USA), which amplify a 268-bp segment of the β-globin gene.

PCR methodology

One and a half micrograms of phenol–chloroform-extracted DNA or 5 μl of digestion buffer-extracted DNA were analysed. PCR reactions were performed in 50-μl volumes including 1 × PCR buffer (50 mM potassium chloride, 10 mM Tris-HCl pH 8.4, 2.5 mM magnesium chloride), 200 μM of each deoxynucleotide (Pharmacia, Uppsala, Sweden), 25 pmol of each primer and 1.25 U of *Taq* polymerase (Gibco, Gaithersburg, MD, USA).

The first round of the nested PCR amplification was performed for 30 cycles in a Perkin Elmer Cetus Thermal Cycler 480 with the outer primers 5′-ACCTGAGGAGACGGTGACC-3′ for the J_H region and 5′-CAGCCTTGAAACATTGATGG-3′ for MBR (Pharmacia Biotech, Roosendaal, The Netherlands). Each cycle included 45 s of denaturation at 95°C, 1 min of annealing at 60°C and 2 min of extension at 72°C. A touch-down PCR technique was used starting from an annealing temperature of 60°C, which was decreased every other cycle by 1°C for the first eight cycles.

Five microlitres of the first-round DNA product was reamplified for 30 cycles with inner primers 5′-CAGGGTTCCTTGGC-CCCAG-3′ for the J_H region and 5′-AGTTGCTTTACGTGGC-CTGT-3′ for the MBR (Pharmacia Biotech). Each cycle included 45 s of denaturation at 94°C, 1 min of annealing at 57°C and 1 min of extension at 72°C.

DNA amplification was always started with 10 min of initial denaturation at 95°C and finished with 10 min of final extension at 72°C.

Patient samples were always analysed together with the human B-cell lymphoma cell line DoHH2 (provided by Dr FE Cotter, London, UK) as positive control and the reaction mixture with no DNA as negative control. The appropriate procedures to avoid contamination were always followed.

Ten microlitres of the second-round product were loaded in 2% agarose electrophoresis gel containing ethidium bromide and visualized under ultraviolet light.

Statistical methods

Overall survival (OS) was defined as the time from the day of diagnosis until death from any cause or until the last follow-up. Time to progression (TTP) was measured as the time from the diagnosis until progression, relapse after response or death from lymphoma. Actuarial survival probabilities were calculated using the life-table method. Survival curves were estimated using the Kaplan–Meier method and differences were evaluated using the log-rank test. The STATA software package (Computing Resource Center, Santa Monica, CA, USA) was used for all statistical procedures.

RESULTS

After PCR analysis of peripheral blood samples, 35 of 59 (59%) cases of FL were bcl-2(MBR)/J_H positive and 24 (41%) were negative at diagnosis. Patient characteristics are listed in Table 1. The two groups had similar clinical, biological and therapeutic characteristics with the exception of the histological subtypes: the number of bcl-2(MBR)/J_H-positive patients was significantly higher in the follicular predominantly small cleaved cell lymphoma subgroup (IWF-B).

The median follow-up time was 46 months. For the group as a whole, the projected 5-year OS was 83% (95% CI 68–91%) and the 5-year TTP 39% (95% CI 23–55%).

The presence of the bcl-2/J_H rearrangement in the peripheral blood at diagnosis was associated neither with a significantly worse OS ($P = 0.751$) nor with shorter TTP ($P = 0.867$).

Among the prognostic factors analysed, ECOG PS > 1 was significantly associated with reduced OS ($P < 0.0001$) and TTP ($P < 0.0001$); age > 60 years and liver involvement were significantly associated with worse OS ($P = 0.0003$ and $P = 0.0352$ respectively). High-risk according to the IPI was associated with worse OS and TTP (both $P<0.0001$).

For 17 patients who had a bcl-2/J_H rearrangement detectable at diagnosis in both peripheral blood and bone marrow, peripheral blood PCR assay was performed after treatment. Fourteen patients (82%; 95% CI 56–96%) became bcl-2/J_H negative; 9 of these 14 patients were further analysed during follow-up and circulating cells carrying the bcl-2/J_H rearrangement reappeared in five of them after several months (median 16, range 7–28 months). Five patients remained negative after a median follow-up of 23 months (range 16–43 months). Clearance of circulating bcl-2/J_H-positive cells was correlated with better OS ($P = 0.04$) but not with longer TTP.

DISCUSSION

Bcl-2 protein is a potent blocker of programmed cell death (Hockenberry et al, 1990).

More than 90% of FL overexpress bcl-2 protein because of the t(14;18) translocation. The clinical significance of the detection of this translocation by PCR is debatable.

Some authors (Johnson et al, 1995; Whang-Peng et al, 1995) show a better clinical outcome for t(14;18)-positive cases, while others (Pezzella et al, 1992; Tilly et al, 1994) fail to demonstrate any prognostic significance for either both *bcl-2* gene rearrangement or bcl-2 protein expression.

Our series of 59 cases confirmed the absence of prognostic significance of bcl-2(MBR)/J_H detection by PCR at diagnosis. These data are not surprising as the majority of the patients with FL have circulating t(14;18) B-cells (Gribben et al, 1991; Berinstein et al, 1993; Lambrechts et al, 1993), and even healthy people may harbour translocated clones (Liu et al, 1994; Bell et al, 1995; Ji et al, 1995; Limpens et al, 1995; Dolken et al, 1996).

Even the significance of 'molecular remission', after both conventional or myeloablative chemotherapy, is a matter of debate. Price et al (1991) and Finke et al (1993) report the presence of t(14;18)-positive circulating cells in patients in long-term complete remission after conventional chemotherapy. Lambrechts et al (1994) show no correlation between the presence or absence

of peripheral blood positive cells and clinical outcome. Gribben et al (1994) report that the reappearance of circulating positive cells is strongly associated with relapse after myeloablative therapy. Similar results are shown by Hardingham et al (1995). Recently, 50–70% of cases with peripheral blood clearance have been reported after non-myeloablative therapy, both in responders and in non-responders (Betticher et al, 1996; Cabanillas et al, 1996).

In our series, positive cases with clearance of circulating bcl-2/J_H-positive cells appeared to have a better OS but not a longer TTP. However, because of the prolonged natural course of the disease, a longer follow-up is necessary before any definitive conclusion.

Peripheral blood is the most easily accessible tissue for follow-up analysis of bcl-2/J_H rearrangements. Our study suggests that the loss of t(14;18) detection by PCR in the blood bodes well for survival. It may be concluded that post-treatment examination of the peripheral blood in FL by PCR may have clinical relevance in predicting the survival pattern of patients and for monitoring response to treatment. However, the use of a standardized PCR method would be needed in the future to allow comparison of results from different institutions, as current data on the clinical value of molecular remission are still controversial.

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