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# Diagnostic Value of the Detection of t(14;18) Chromosome Translocation in Malignant Hematological and Immunopathological Diseases Using Polymerase Chain Reaction

István TAKÁCS, Margit ZEHER, László URBÁN, Gyula SZEGEDI and Imre SEMSEI*

Department of Internal Medicine III, Molecular Biology Research Laboratory, University of Debrecen, H-4004, Hungary

The majority of the t(14;18) chromosome translocations that occur in non-Hodgkin centroblastic-centrocytic follicular lymphoma can be detected by various methods. During the translocation process the *bcl-2* gene located on chromosome 18 (18q21) is translocated to the $J_H$ region of the immunoglobulin gene of chromosome 14 (14q32). The most frequent type of *bcl-2* translocations is the *mbr* type, whereas the immunoglobulin gene breaks mainly at the $J_{H1-6}$ exons. About one of the $10^5$ cells bearing the translocation can already be detected by using nested polymerase chain reaction (PCR). Eight patients suffering from follicular lymphoma were included in this study, which considered the usefulness of the PCR method. The results are in good agreement with those obtained by conventional diagnostic methods. Translocation can be detected, however, in patients with non-malignant diseases such as Sjögren's syndrome (about 5% of the patients) and in a patient with Whipple disease. In addition, translocation was detected in lymphocytes of peripheral blood of a healthy donor. Since lymphomas are detected in patients with Sjögren's syndrome with a relative high frequency, an early diagnosis of the translocation could improve the treatment of the disease. Nevertheless, a diagnosis of lymphoma is valid only in cases of bone marrow translocation-positivity.

**Key words:** t(14;18) chromosome translocation, lymphoma, early diagnoses, Sjögren's syndrome

The most frequent tumor type associated with B-lymphocytes is follicular lymphoma (FL) (1) and this type represents 1/2 of the total percentages of the non-Hodgkin lymphomas (NHL) (2, 3). Both the histological appearance and the clinical process of FLs can correspond to low- and high-malignity NHL. Cytological grades I, II, and III can be assigned according to the "REAL" classification (4, 5).

Approximately 60% of FLs are characterized by the presence of t(14;18) chromosome translocation (6), that translocation involves the $J_H$ segment of the immunoglobulin heavy chain on chromosome 14 (14q23.3). Remarkably, that same segment is rearranged during the development of B-lymphocytes. It is supposed that the translocation occurs because of a mistake in $V_H$-D or D-$J_H$ joining (7); this suggestion is supported by the fact that the part between the $D_H$ and $J_H$ regions disappears during translocation (8). The immunoglobulin heavy chain also plays a role in the formation of Burkitt lymphomas, mouse plasmocytomas (9) as well as in certain B-cell lymphomas (10), however, in the latter case the *bcl-1* gene is translocated from chromosome 11. In the case of FLs the *bcl-2* gene is translocated from chromosome 18 (18q21.3). The break points on the *bcl-2* gene are concentrated in 3 regions, the major breakpoint region (*mbr*), minor cluster region (*mcr*), and variable cluster region (*vcr*) (Fig. 1). The *mbr* is a 500 base pair (bp)-long region at the 3′ non-translated part of the *bcl-2* gene (11), and the *mcr* involves a gene section 30 kb downstream of the coding region. The *vcr* breaks occur in the 5′ non-translated part of the *bcl-2* gene; they account for only a few percentages of the translocations, whereas the *mbr* region comprises 3-quarters of it.

In addition to its occurrence in lymphomas, the presence of the t(14;18) translocation can be revealed in other diseases, *e.g.*, in certain autoimmune diseases, in reac-

* To whom correspondence should be addressed.

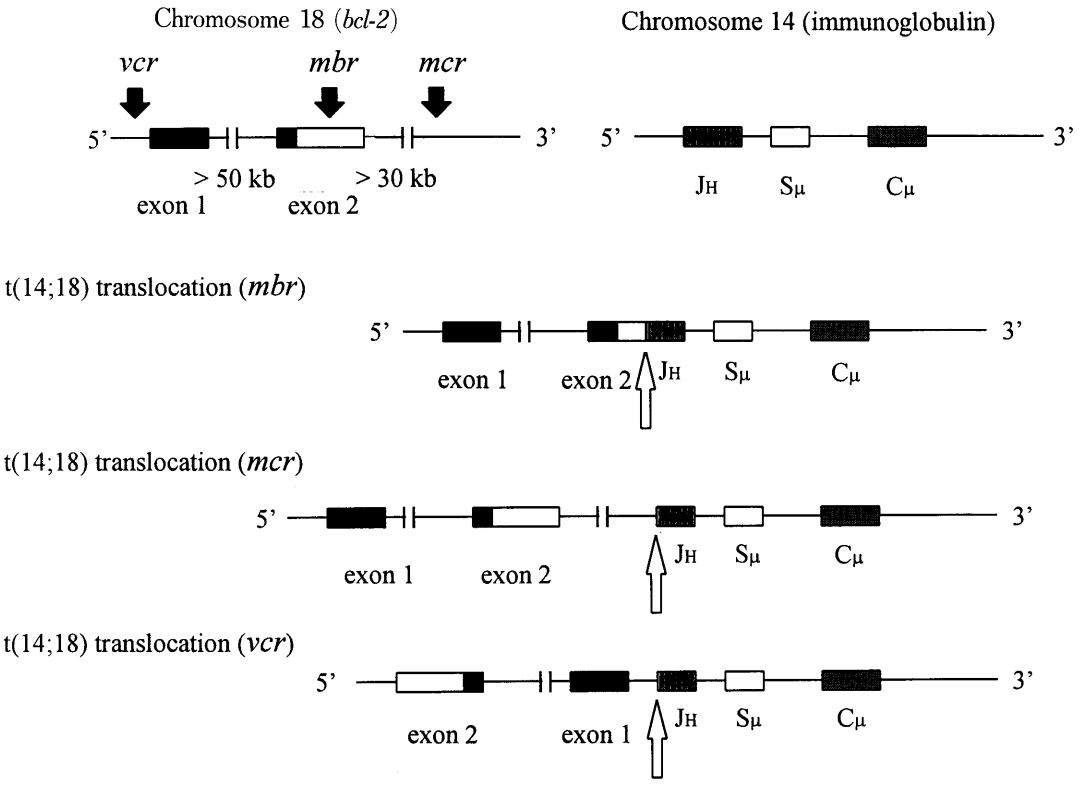


**Fig. 1** A schematic representation of the 3 different types of t(14;18) translocation. *mbr*, major breakpoint region, *mcr*, minor cluster region; *vcr*, variable cluster region. The open arrows indicate the joining of the 2 chromosomes.

tive lymph nodes and even in the lymphocytes of peripheral blood of healthy donors. In the case of Sjögren's syndrome (SS) the relative high frequency of the appearance of translocation parallels the well-known fact that the incidence of lymphoma is much more frequent in patients with SS, compared to the incidence rate in healthy donors.

About 5% of the bone marrow infiltrations can be recognized by conventional morphological investigations of neoplasms. Other methods (*e.g.*, flow cytometry, Southern blot analysis and the clonal excess method) can detect about 1% of the translocations (12). However, the most suitable method for such detection is the polymerase chain reaction (PCR) technique (13, 14), which can reveal translocation in 1 of $10^5$ cells. Amplified sequences can be detected in most cases using a simple agarose gel; this technique involves ethidium bromide and ultra violet (UV) light. The sequences can also be detected by further hybridization with a labeled cDNA probe or by flow cytometry (15). "Nested-PCR" was used for the present experiments, in which a second amplification was performed after amplification using additional internal primers had been initiated. Thus, a very sensitive and highly specific amplification can be conducted.

In this paper the detection of t(14;18) translocation and its significance are described in patients with FL (13), SS (16), Whipple disease (17) as well as in healthy donors.

## Patients and Methods

***Lymphoma patients.*** No. 1, Mr. Cs. B. (62 y.): His NHL (centroblastic-centrocytic: CB-CC) was diagnosed in March, 1995. The bone marrow and the skin had already been infiltrated by the time of diagnoses. This patient did not have, however, pathologically enlarged lymph nodes. The histological diagnosis was established based on skin biopsies and a bone marrow sample. Clinical stage: IV/A (Ann-Arbor).

No. 2, Mr. J. B. (56 y.): During physical examination of this patient, bean-size, painless lymphatic glands were noticed axially and supraclavicularly. Gland excision

revealed a malignant B-cell lymphoma with follicular centroblasts with anaplastic centrocytic elements. Tumor cell infiltration was not observed in the medulla-smear. Radiological examinations (Computer Tomography, ultrasound) did not show enlarged lymph nodes, either in the hilus of the lung or in the retroperitoneum. The peripheral blood proved to be normal, and fluorescence cytofluorimetry did not reveal lymphomal infiltration. Clinical stage: II/A (Ann-Arbor).

No. 3, Ms. SZ. J. (62 y.): This patient's FL had already been diagnosed in 1992 (NHL, CB-CC). She received chemotherapy by which remission was established. In the middle of 1996 she relapsed in the hilus and retroperitoneum; therefore a new protocol was initiated. Clinical stage: III/A (Ann-Arbor).

No. 4, Mr. Cs. B. (80 y.): This patient has suffered from NHL since 1966. Enlarged lymph nodes in the hilus, retroperitoneum, and on both sides of the neck and axilla were present. A sternum puncture did not show lymphoid infiltration. Clinical stage: III/A (Ann-Arbor).

No. 5, Ms. L. L. (72 y.): Her FL of stage III/A was diagnosed in the hilus and retroperitoneum. Her general condition was characterized by bad cardio-pulmonar function. Chemotherapy was started, but the patient died of severe infection.

No. 6, Mr. E. I. (55 y.): This patient's lymphoma was staged as III/A at the time of diagnoses. He proved to be resistant to the therapy and he died soon after chemotherapy was started.

No. 7, Ms. T. I. (72 y.): She has suffered from SS since 1987; lymphadenomegaly was observed in 1993. Examination of this patient revealed a diffuse Hodgkin's lymphoma and she recieved chemotherapy. In the middle of 1996 a biopsy showed an anaplastic large cell malignant lymphoma. Clinical stage: III/A (Ann-Arbor). Following cytostatic treatment, the lymph nodes regressed.

No. 8, Ms. V. A. (66 y.): This patient's NHL (CB-CC) was diagnosed in late 1977. Her hematological disease dominated the retroperitoneum, both kidneys were infil-trated, furthermore, lymphadenomegaly was established in the neck. Examination of the bone marrow showed no disease. Clinical stage: III/B/E (Ann-Arbor).

***Patients with SS.*** Ten of the 400 SS patients of our clinic (15, 18) were included in this study. Peripheral lymphocytes were separated in a clinically inactive period; the presence of lymphoma in the patients studied was excluded at that time on the basis of the clinical examinations.

***Other patients and donors.*** In addition to the histological and clinical symptoms that characterize the Whipple-disease (19), our 42-year-old female patient is allergic to certain medicines, and shows autoantibody positivity, hypertony, helicobacter-positive gastritis as well as insulin dependent diabetes mellitus. Symptoms of lymphoma were not observed in this patient.

Lymphocytes of healthy donors were taken from individuals who did not have any symptoms of disease.

***DNA isolation.*** Lymphocytes were isolated from the peripheral blood and medulla by standard Ficoll gradient centrifugation (20). The DNA was isolated for PCR by detergent and proteinase K digestion (21).

***PCR.*** Nested-PCR was performed as described in previous reports (22-25), with the following modifications: 1 $\mu$g DNA was amplified using Amplitaq polymerase (Boehringer) in a final volume of 25 $\mu$l in Perkin Elmer 9600 equipment. Cycle-conditions of 94 °C, 15″; 55 °C, 30″; and 72 °C, 30″ were applied. The first 30 cycles were carried out using the following primers: *mbr1*: 5′-AAATCTATGGTGGTTTGACCTTTAGAG-3′ (the external primer of *bcl-2*, *mbr* region) and J6: 5′-AACATGGTCCAGTCCGCCAGGTC-3′ (external primer for the immunoglobulin). The next 30 cycles were performed, from 0.5 $\mu$l samples taken from the first amplification, using the following primers: *mbr2*: 5′-GAGTTGCTTTACGTGGCCTGTTTC-3′ (inner primer for the *mbr* region) and JCO1: 5′-(AT) (TA) CT (TC) ACCTGAGGAGACGGTGACC-3′ (the consensus primer of immunoglobulin J exons). The results of the amplifications were made visible under UV light after agarose gel (3%) electrophoresis and ethidium bromide staining. Results were documented by a Polaroid instant camera. The sensitivity of this type of PCR is about 1 cell carrying the translocation out of $10^5$ cells. This sensitivity has been measured by other laboratories under similar conditions used in the present study.

***Sequencing.*** The PCR products were isolated from low melting point agarose and then they were inserted into a pCR 1,000 vector by a TA cloning system (Invitrogen). The PCR products were then transformed into *E. coli* and they were isolated by Magic miniprep (Promega). The sequences were determined by using a Sequenase 2.0 system (US Biochemicals), and then the GeneBank database was used for sequence analysis.

## Results

The results obtained after studying a patient (No. 1) suffering from FL are shown in Fig. 2A. Columns 2 and 3 indicate characteristic negative results, *i.e.*, without any translocation present. A non-specific "germline" band can be observed at about 500 bp and a fainter band can be seen at 220 bp (this latter line is detectable even in the ninth column, which shows a control reaction made without DNA.) In the fourth column there are 2 very strong, specific lines at about 210 and 170 bp; these lines indicate that translocations occurred in the *mbr* region of the *bcl-2* gene of the lymphocytes of peripheral blood. The upper line was also well amplified from the lymphocytes separated from the medulla (column No. 5). However, the lower line is barely visible. The 210 bp DNAs amplified in both columns were sequenced. Fig. 2B shows that the sequences obtained were completely identical in both columns. The primers applied (*mbr2* and JCO1) surround the amplified pieces of *bcl-2* and immunoglobulin DNAs. Between these 2 DNA pieces there is a sequence of the N region that usually can be found in the translocations (ranging from 1 to a few 10 nucleotides). The 150 bp of the *bcl-2* gene, 10 bp of the N region and the 46 bp of the immunoglobulin result in a 206 bp-long amplified DNA. We have sequenced many more PCR-amplified products and all of them proved to be translocations indicating the specificity of our method.

One month after the therapy was initiated translocation could not be amplified either from lymphocytes of peripheral blood (column 7) or from those of bone marrow (column 8). These findings indicate that the number of translocation-bearing cells is under one of approximately $10^5$ cells. (Nevertheless, none of the other methods were able to diagnose the presence of the translocation, if the number of translocation-bearing cells fell below $10^5$). Thus, one can notice a remission after 1 month of the therapy.

Fig. 3 shows the results of amplifications from the peripheral blood of 8 patients suffering from FL (Nos. 1 -8). All of these patients were treated, and the incidence of remissions and relapses was noted. Fig. 4 indicates the results yielded during the follow-up period after treatment of these patients. Three of the patients have died due to generally bad physical condition caused, for example, by alcoholism. It can be seen, however, that as a results of the treatment there are relapses, which were in some cases followed by remissions. The results of PCR detection were

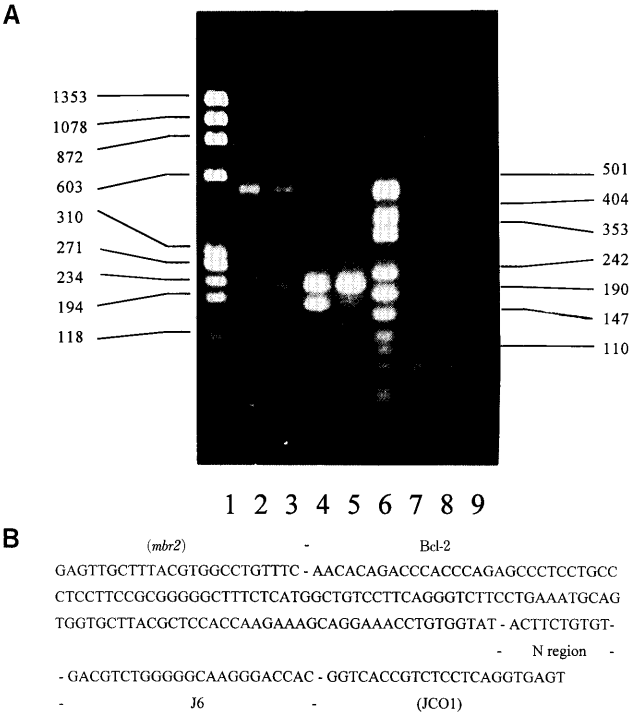


**Fig. 2** Detection of the t(14;18) chromosome translocation in follicular lymphoma.
**A**, results of DNA amplifications from lymphocytes isolated from different sources using nested-polymerase chain reaction. 1, ΦX174 DNA marker in base pairs; 2, control peripheral lymphocytes; 3, control lymphocytes of bone marrow; 4, peripheral lymphocytes of a follicular lymphoma patient; 5, bone marrow lymphocytes of a lymphoma patient; 6, pUC18 DNA marker; 7 and 8, results yielded 1 month after the therapy of the lymphoma patient as in columns 4 and 5 respectively; 9, amplification reaction without DNA.
**B**, sequence of the amplified DNA (upper lane of column 4 of Fig. 2A). *mbr2* and JCO1 are the inner primers of the *bcl-2* and immunoglobulin genes respectively. A portion of the *bcl-2* gene and J6 exon of the immunoglobulin gene can be seen between the 2 primers, together with the nucleotides of the N region.

in good agreement with those obtained by conventional diagnostic methods.

Not only patients with FL but also patients suffering from other diseases have translocation-bearing cells. Fig. 5A illustrates that the patients with SS, one Whipple disease patient, and one healthy donor proved to be translocation-positive as regards lymphocytes of peripheral blood. Columns 1 and 2 show the presence of the translocation in 2 of the patients with SS. Fig. 5B shows the amplified sequence of column 2, proving the specificity of the amplification.

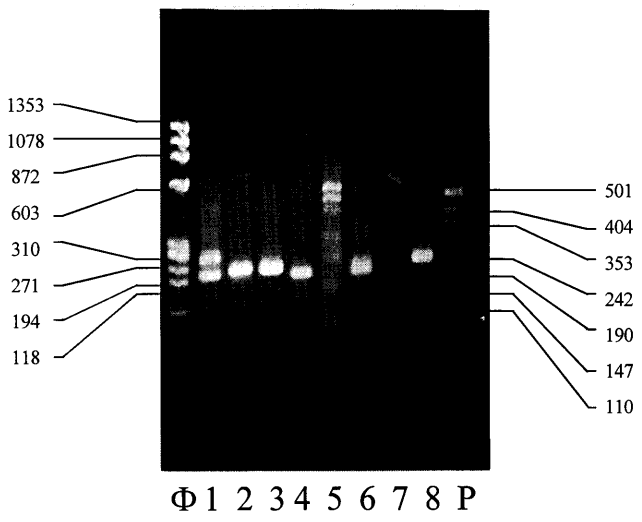


**Fig. 3** Translocations of various patients with follicular lymphoma. Numbers 1-8 represent the patients number 1-8 of Fig. 4. Patients 5 and 7 were translocation-negative at the time of the measurement in their peripheral lymphocytes.

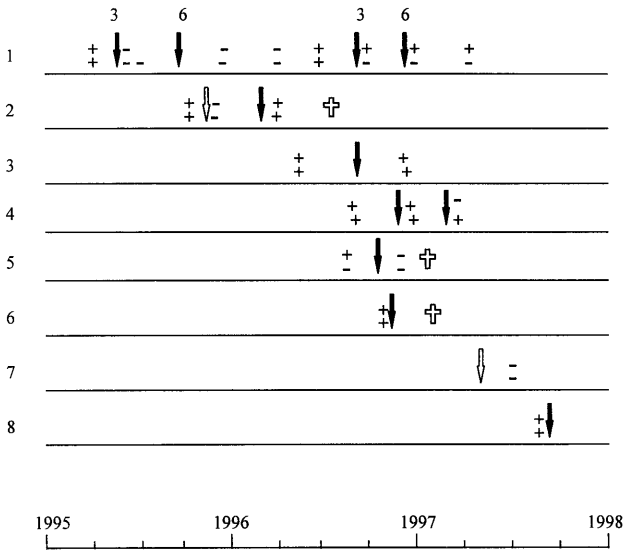


**Fig. 4** Follow-up of the presence of t(14;18) translocation in patients with follicular lymphoma. +, translocation is present; −, translocation is absent. The upper lane indicates result yielded from peripheral lymphocytes, and the lower one shows the results yielded from the lymphocytes of bone marrow. Black arrows, chemotherapy; open arrows, radiotherapy treatments. White cross represents the death of the patient.

## Discussion

Our nested-PCR method proved to be a sensitive and specific diagnostic tool for the detection of t(14;18) chromosome translocation. However, the detection of translocation is not the only application of this method in this context; follow-up study after treatment of patients suffering from FL is also improved by this method. All of our PCR results were in good agreement with results obtained by conventional diagnostic measurements. In addition, all of the relapses and remissions detected by other methods were validated by PCR. This nested-PCR method is also helpful for differential diagnostics, in cases, in which results yielded by conventional measurements are not sufficient for establishing a correct diagnosis. In the case of patient No. 2, if the PCR results had been taken into consideration, chemotherapy would have been applied instead of radiotherapy, even for the initial treatment. This patient was categorized with stage II/A (Ann Arbor (26, 27)), hence only radiotherapy was administered. However, the results of PCR indicated a more severe stage of illness. Therefore, this patient should have been categorized as having a IV/A-stage lymphoma, and chemotherapy should have been administered from the onset of treatment.

It is well documented that healthy donors can bear a transient translocation for several months (28, 29), and that the frequency of such translocations increases with age (30). It is thus not surprising to find such donors on the occasion of a local inflammation process (29). In addition, the appearance of SS also elevates as a function of senescence (31), which is parallel to the increased frequency of lymphoproliferative and other malignant diseases (32). These illnesses are the cause of death for most patients with SS (33); therefore, presence of t(14;18) translocation can be inferred. Indeed, about 5% of all patients with SS carry the translocation, as it is reflected in our results (46). However, not only the patients with SS, but one of our 2 patients with Whipple diseases (19) proved to be t(14;18) translocation-positive. It has already been reported of a patient with Whipple disease that carried *bcl-2* rearrangement (34) but not t(14;18) translocation. The causes of such rearrangements remain unclear; however, lymphomas are known to be associated with the disease (35).

Relatively high portion of the patients with SS has lymphomas, as compared with the healthy population (36-38). Among our patients suffering from SS (n = 400), the incidence of NHL was 2.2% (39). A higher percent

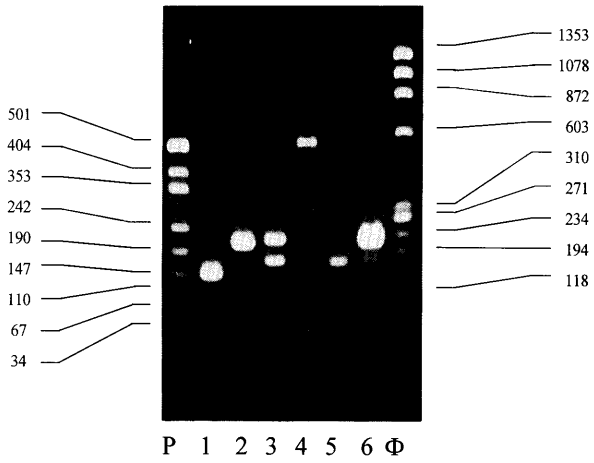


**Fig. 5** Presence of the t(14;18) translocation in peripheral lymphocytes of various sources.

**A**, picture of the results of polymerase chain reaction amplifications. P, pUC18 DNA standard; 1 and 2, patients with Sjögren's syndrome; 3, patient with follicular lymphoma (DNA was isolated after chemotherapy); 4, negative control; 5, patient with Whipple disease; 6, "healthy" donor; Φ, ΦX174 DNA standard.

**B**, sequence of the amplified DNA of column 1 of Fig. 5A. *mbr 2* and JCO1 are the appropriate primers of the *bcl-2* and immunoglobulin genes. The amplified parts of the *bcl-2* and immunoglobulin genes can be seen, together with the N region.

of our Sjögren's patients are t(14;18) translocation-positive; however, none of these patients was diagnosed with a lymphoma (unpublished data). A few of these patients did bear the translocation, even in the lymphocytes of the bone marrow. Therefore, such patients might be predisposed to develop follicular lymphoma over a long time course (47). The advantage of PCR is that translocations can be detected at early stages; this increases the chance of obtaining an early diagnosis of lymphoma. FL is the result of a long latent period during which only the peripheral lymphocytes carry translocation-bearing cells; these cells in turn infiltrate the bone marrow, but only later on. The frequently occurring inflammations, together with other factors, could result in translocation-positive lymphocytes in the Sjögren's patients, in turn the translocation-positive lymphocytes could induce a process that results in lymphoma. It is surprising, however, that the translocation-positive patients do not have enlarged salivary glands, which is a predisposition of a malignant lymphoma (25% of our patients have the enlargement) (16, 18). This finding indicates that translocation-positivity is an early marker, even when the characteristic clinical symptoms are still absent. It will be important in the future to investigate such patients using clinical and molecular biological methods; hopefully such study will continue to yield more insights into the appearance of lymphoproliferative diseases.

In cases of FL the t(14;18) translocation causes an alteration of the *bcl-2* expression (40–42), which in turn leads to a disturbance of the apoptotic process. The over-expression of the Bcl-2 protein may lead to mono- and polyclonal lymphoproliferation; this process is enhanced by an alteration of the fas gene as well (43). The latter process leads to the disturbance (44) of the normal metabolic and apoptotic processes (45). All of these factors, when considered together, provide a deeper insight into the etiology of autoimmune and/or lymphoproliferative syndromes.

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