ORIGINAL PAPER

Prognostic and predictive significance of the bcl-2/IgH translocation in malignant follicular lymphomas

ALINA GEORGESCU[1)], M. STOICEA[1)], MARIA COMĂNESCU[1)], CAMELIA DOBREA[1)], F. ANDREI[1)], MARIA NEAGU[1)], FLORINA CIONCA[1)], ANCA CIOBANU[2)], ANCA LUPU[2,3)], CARMEN ARDELEANU[1,3)]

[1)]*Department of Pathology, "Victor Babeş" National Institute of Pathology, Bucharest*

[2)]*Department of Hematology, "Colţea" Clinical Hospital, Bucharest*

[3)]*"Carol Davila" University of Medicine and Pharmacy, Bucharest*

Abstract

Background: The t(14;18) translocation, which leads to an overproduction of the bcl-2 protein, supposedly occurs in almost all follicular lymphomas (FL) and can be detected by FISH methods or by PCR. Its detection is useful in monitoring the response to therapy and in assessing minimal residual disease in bone marrow. Recently it was observed that the translocation could become negative after treatment. The prognostic and predictive significance of this fluctuation is not entirely understood. *Aim*: We intended to find significant correlations among morphological features, histological grades, immunohistochemical findings, and cytogenetical aberrations in malignant follicular lymphomas, in order to identify the prognostic and predictive value of the bcl-2/IgH translocation in these malignancies. *Material and Methods*: We conducted a study on 79 patients with follicular lymphomas. The study was carried out on tissue samples selected from the "Victor Babes" National Institute of Pathology files. These samples were tested by immunohistochemistry and FISH. *Results*: Most of the cases (65.2%) were low-grade FL (grade 1–2). Approximately 58.8% of cases in the FISH study group presented t(14;18). In 66.6% of the cases with t(14;18), the immunohistochemical reaction for bcl-2 protein was positive. A significant positive correlation was found between the IHC positivity for bcl-2 and t(14;18) detected by FISH (p=0.04). *Conclusions*: Bcl-2 t(14;18) plays an important role in the pathogenesis of follicular lymphoma. FISH is an important tool in the diagnosis, treatment and follow up of these malignancies, since the immunohistochemical testing is negative in a significant proportion of cases.

***Keywords*:** follicular lymphoma, t(14;18), bcl-2, FISH.

Introduction

Follicular non-Hodgkin lymphomas have an increasing incidence, but a decreasing age of patients at onset. Most frequently, they present as generalized lymphadenopathy and bone marrow involvement in middle-aged and elderly individuals, with a median age at diagnosis of 55–65 years and a various course, from rather indolent to disseminated and rapidly growing disease [1].

In Europe, follicular lymphomas constitute up to 30% of non-Hodgkin lymphomas [2]. In spite of their indolent character, the overall survival is not influenced by classical treatment and a significant number of patients are dying within two years [3]. Follicular lymphomas are difficult to cure because of frequent recurrence [4]. Patients who achieve complete clinical remission usually relapse on the account of neoplastic cells that survive below the limits of detection using the current standard techniques.

Prognostic markers, identified by different working groups, can be classified as: *morphological* (histological grade, proliferation rate, diffuse areas, sclerosis, microenvironment cells, minimal residual disease) [3, 5], *immunohistochemical* (Ki67 index, CD20, bcl-2 and bcl-6 positivity) and *molecular* (the bcl-2/IgH hybrid gene, the t(14;18)(q32:q21) translocation).

The t(14;18) translocation, generated through by the bcl-2/IgH rearrangement, supposedly occurs in almost all follicular lymphomas (FL) and can be detected by FISH methods or by PCR in primary lymphomas [4, 6]. Moreover, its detection is useful in monitoring the response to therapy and in assessing minimal residual disease in bone marrow [7].

The t(14;18) translocation leads to an overproduction of bcl-2 protein (an inner mitochondrial membrane protein), known as a potent apoptosis inhibitor [4, 6]. The bcl-2 protein can be overexpressed or absent immunohistochemically. FISH is preferred usually on paraffin embedded material, applied on interphase nuclei [8], by dual-color fluorescence [9]; certain authors recommend it at diagnosis, as equally sensitive and more specific than PCR [10, 11].

Recently it was observed that the translocation could become negative after treatment. The prognostic and predictive significance of this fluctuation is not entirely understood, but some authors observed a positive correlation between post-therapeutic translocation regression

in peripheral blood or bone marrow and prognostic improvement [12].

The aim of our study was to identify possible correlations between morphological features, histological grades, immunohistochemical findings, and cytogenetical aberrations in malignant follicular lymphomas, in order to identify the prognostic and predictive value of bcl-2/IgH translocation in these malignancies.

Material and Methods

We conducted a study on 79 patients with follicular lymphomas. The study was carried out on tissue samples selected from the "Victor Babeş" National Institute of Pathology files. These samples were formalin-fixed paraffin embedded tissues, routinely processed for histology. Sections were cut at 5 μm and stained using the standard Hematoxylin–Eosin (HE) stain.

Immunohistochemistry method

For immunohistochemistry (IHC), paraffin sections were deparaffinized, rehydrated and rinsed in PBS pH 7.4. Retrieval with cooking in specific buffer was raised in microwave oven (Whirlpool) at 800 W for 5 minutes, and 440 W for 10 minutes. The IHC method was an indirect bistadial technique performed with a polymer based detection system (EnVision$^+$ Dual Link System–HRP, Dako, Carpinteria, CA) according to the manufacturer's instructions. All specimens were counterstained with Meyer's Hematoxylin, examined, and photographed with a Nikon Eclipse 600 microscope. The cases were tested by immunohistochemistry (IHC) using monoclonal antibodies against: bcl-2 (clone 124) (dilution 1:50), and CD45RO (clone UCHL1) (dilution 1:50), bcl-6 (clone PG-B6p) (dilution 1:50) (Dako, Carpinteria, CA, USA); CD10 (clone 56C6) (dilution 1:100), CD20 (clone L26) (dilution 1:50) (Novocastra, UK). For negative control, the identical procedure was performed without the primary antibody. As positive controls, we applied the same method on different human lymphoid tissues known to be positive for the aforementioned antibodies.

Fluorescence *in situ* hybridization technique (FISH)

We employed the PathVysion LSI IGH Spectrum Green/ LSI bcl2 Spectrum Orange (VYSIS) kit. The protocol (Table 1) used 4 μm sections from paraffin blocks on distilled/deionized water, that were subsequently placed on electrostatic, specially treated (Silane) slides. The initial stage consisted in deparaffinization (HemoDe, xylene), followed by the complete removal of the solvent with alcohol. The sections were then immersed in HCl 0.2 N for 20 minutes and subjected to a pretreatment solution at 80^{0}Celsius for 30 minutes (a protein digestion phase meant to expose the genetic material). Intermediate washes were performed in buffer solution; before each stage, the slides were dried in hot air jet, at 40–45^{0}C, for 2–5 minutes. Enzymatic digestion using proteinase K was performed at 37^{0}C for 6 minutes. Air-dried sections were dehydrated in successive alcohol baths at increasing concentrations (70%, 85% and 100%), and air-dried again in hot air jet, at 40–45^{0}C, for 2–5 minutes. The next stage consisted in denaturing (5 minutes at 72^{0}C) and hybridization with Path Vysion probes on the HYB plate (14–18 hours at 37^{0}C). Post-hybridization wash ("stringency wash") was performed the following day, at 72^{0}C, for 2 minutes, with buffer solution (pH 7–7.5); the slides were then air-dried in the dark and finally stained with DAPI (4,6-diamino-2-phenylinodole, 10 μL) for chromatin visualization. The slides can be stored in the dark, at -20^{0}C, for a long time. They were analyzed under a Nikon epifluorescence microscope, with a blue filter for DAPI and filters specific for the fluorochrome probes used. The actual assessment consisted in detecting and evaluating green (bcl-2) and orange-red (IgH) signals, single and fused (yellow). Many microscopic fields were examined in each case, since not all cells are positive for the t(14;18)(q32:q21) translocation. The presence of a fused signal, together with single red and green signals, was considered positive and identified the nuclei carrying the bcl-2/IgH rearrangement.

Table 1 – ***FISH protocol***

FISH protocol		
1. Deparaffinization Xylene, 10 minutes Xylene, 10 minutes Ethanol 100%, 5 minutes Ethanol 100%, 5 minutes Hot air jet drying, 45–50^{0}C, 2–5 minutes	2. Pretreatment HCl 0.2 N, 20 minutes Pure water, 3 minutes WB, 3 minutes ×2 Pretreatment solution, 80^{0}C, 30 minutes Pure water, 1 minute WB, 5 minutes Repeat WB, 5 minutes	3. Proteinase K Digestion Remove the WB excess Proteinase K, 37^{0}C, 6 minutes WB, 5 minutes Repeat WB Hot air jet drying, 45–50^{0}C, 2–5 minutes
4. Alcohol dehydration Ethanol 70%, 1 minute Ethanol 85%, 1 minute Ethanol 100%, 1 minute Hot air jet drying, 45–50^{0}C, 2–5 minutes	5. Denaturing and hybridization on the HYB plate Denaturing at 72^{0}C, 5 minutes Hybridization at 37^{0}C, 14–18 hours	6. Posthybridization wash (2nd day) Post HYB preheating bath at 74^{0}C Removal of the covering glass Wash in post HYB buffer (pH 7–7.5) at 74^{0}C, 2 minutes Drying, mounting in DAPI Visualization in fluorescence microscope

Statistical analysis

Statistical analysis has been done using the Student *t*-test, from the Analysis Tool Pack of Microsoft Excel 2003, running under Windows XP Professional. A value of $p<0.05$ was considered significant.

Results

The study group comprised 79 cases. Three cases were excluded after microscopic and immunohistochemical examination, being considered reactive processes. Seventy-six cases were diagnosed as follicular non-

Hodgkin lymphomas. There were 33 male patients (43.42%) and 43 female patients (56.57%). The mean age was 57.82 years (age range 23 to 81 years) for male and 55.24 years (age range 30 to 82 years) for female patients; the study group mean age was 56.53 years.

The most important challenge in histopathological examination was the distinction of follicular lymphomas from reactive lymphadenitis and other types of lymphoma. The reactive processes encountered were characterized by a low density of follicles, the presence of polarity and of a polymorphic appearance within the follicles. In the follicular lymphomas examined histopathologically, we detected nodal architecture effacement by closely-packed follicles with a rather monomorphic appearance, composed of a mixture of centrocytes and centroblasts. Interfollicular infiltrates of neoplastic cells constituted an important diagnostic feature. For histological grading, we used the *WHO Classification* (2008). Most of the cases (65.2%) were low grade FL (grade 1–2) (Figures 1 and 2).

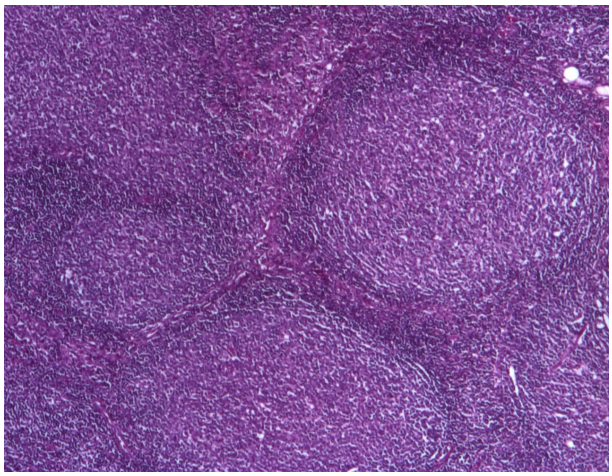

Figure 1 – ***Follicular non-Hodgkin lymphoma grade 1, with diffuse and follicular pattern (HE stain, ob. 4×).***

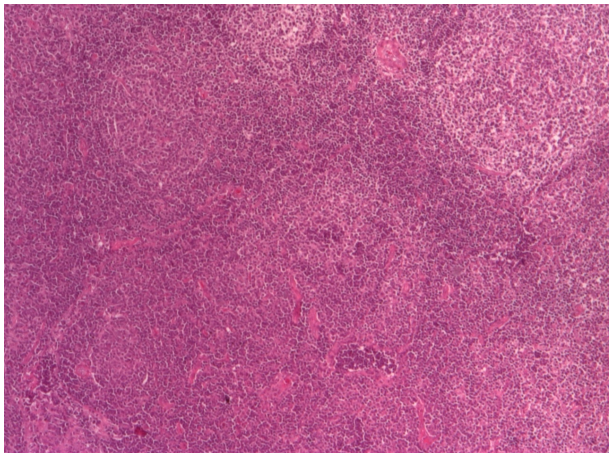

Figure 2 – Follicular non-Hodgkin lymphoma grade 2 (HE stain, ob. 4×).

Another histological parameter considered was the presence of diffuse pattern areas. In our series, the majority of cases presented a follicular and diffuse pattern.

Minimal residual disease was detected in bone marrow biopsies in six cases treated with CVP (Cyclophosphamide, Vincristine, and Prednisone).

Complete remission was obtained in seven cases treated with R–CHOP: Rituximab–Cyclophosphamide, Hydroxydaunorubicin (Doxorubicin), Oncovin (Vincristine), and Prednisone/Prednisolone) or with R–CHP: Rituximab–Cyclophosphamide, Hydroxydaunorubicin (Doxorubicin), and Prednisone/Prednisolone.

All cases were positive for L26 (CD20) in tumor cells (Figure 3). CD45RO (UCHL1) was positive in reactive small peri-follicular lymphocytes, and negative in tumor cells.

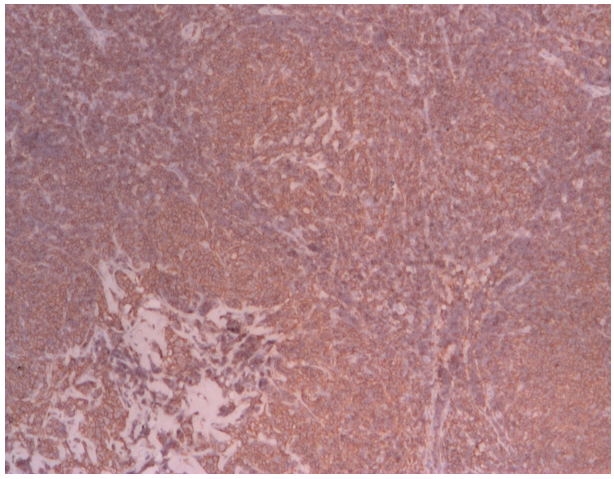

Figure 3 – ***Follicular non-Hodgkin lymphoma grade 2. Diffusely positive IHC reaction for L26 in tumoral cells, ob. 4×.***

The IHC expression of bcl-2 was positive in 76.6% of cases (Figure 4). Bcl-2 negativity was encountered in 23.4% of cases. Forty percent of the bcl-2 IHC positive cases were also bcl-6 positive.

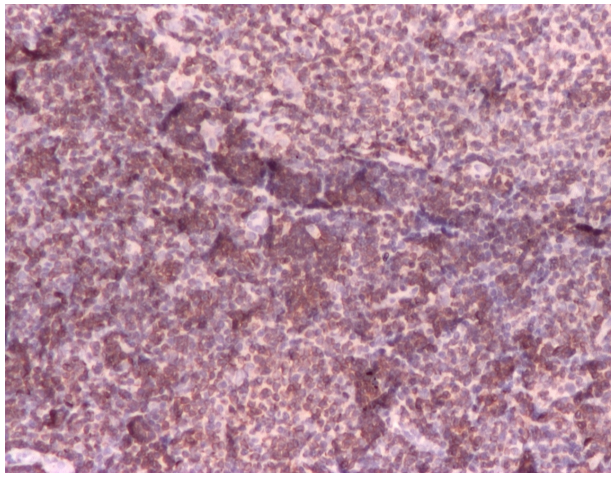

Figure 4 – ***Follicular non-Hodgkin lymphoma grade 2. Positive IHC reaction for bcl-2 in tumoral cells, ob. 10×.***

The IHC expression of CD10 was encountered in 71.5% of cases (Figure 5).

Twenty-two cases were randomly selected for FISH analysis. Five cases were excluded because of processing artifacts, for which hybridization could not be interpreted. Ten of the 17 cases without artifacts (58.8%) presented the t(14;18) translocation: two cases of FL grade 1–2, two cases of FL grade 1, six cases of FL grade 3 (Figure 6).

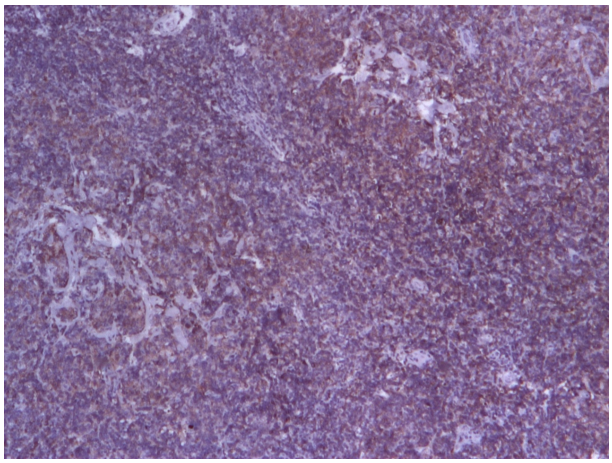

Figure 5 – ***Follicular non-Hodgkin lymphoma grade 2, with diffuse and follicular pattern. Positive IHC expression for CD10 in tumoral cells, ob. 4×.***

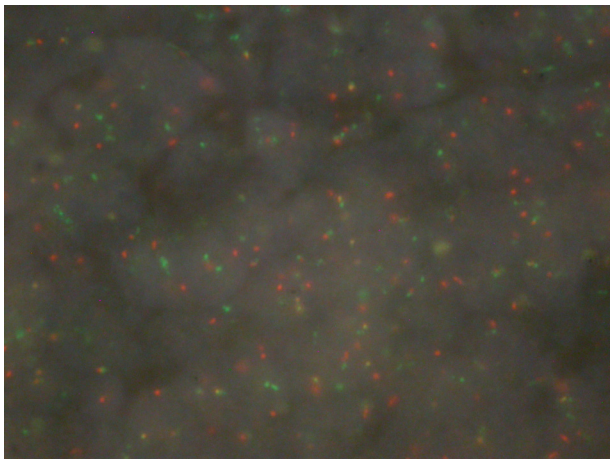

Figure 6 – ***Follicular non-Hodgkin lymphoma grade 1. Interphase FISH pattern of nuclei with t(14;18) translocation exhibiting fusion signals (yellow) in addition to green and red signals (DAPI–Texas Red–FITC, 100×).***

In 66.6% of cases with t(14;18) translocation, the immunohistochemical reaction for bcl-2 protein was positive. The IHC expression of CD10 was positive in 50% of the cases with bcl-2/IgH rearrangement.

Statistical analysis was performed with the Student *t*-test. After excluding the cases with incomplete determination of their immunoprofile, the FISH study group was reduced at 10 cases suitable for statistical analysis. A significant positive correlation was found between the IHC positivity for bcl-2 and the t(14;18) translocation detected by FISH (p=0.04).

Discussion

The study group was randomly selected based on the initial histopathological diagnosis. After revising the series, three cases were excluded as reactive processes. Seventy-six cases diagnosed as follicular non-Hodgkin lymphomas were reclassified both histopathologically and immunohistochemically.

The number of male patients was smaller compared to female patients; a relatively similar mean age was found for both sexes, but a smaller age of onset for male patients (seven years less) was noted.

Histopathological grading was based on the *WHO Classification* (2008): Grade 1 follicular lymhomas (0–5 centroblasts/HPF), Grade 2 follicular lymphomas (6–15 centroblasts/HPF), and Grade 3 follicular lymphomas (>15 centroblasts/HPF). Grade 3 follicular lymphomas were subdivided in two categories: 3a (>15 centroblasts, but centrocytes are still present) and 3b (centroblasts form solid sheets with no residual centrocytes). Histopahological grading found most of the cases (65.2%) were low-grade FL (grade 1–2). However, for the FISH technique, predominantly high-grade FL were selected, due to diagnostic difficulties of the cases referred to the Institute.

Lymph node areas lacking follicles and follicular dendritic cell meshwork were considered as diffuse component. These areas contain more than 15 centroblasts/HPF (grade 3) and can be classified as diffuse large B-cell lymphoma within a follicular lymhoma. In our study group, the diffuse pattern of tumor proliferation was more extensive as usually reported in literature. It was always associated with the classical follicular pattern.

The evaluation of minimal residual disease was done histologically and immunohistochemically on bone marrow biopsies, and was found in six cases treated with CVP (Cyclophosphamide, Vincristine, and Prednisone).

Following the addition of Rituximab to chemotherapy, complete remission was obtained in seven cases treated with R–CHOP or R–CHP. It is known that Rituximab may determine a longer disease free interval [13].

Patients in long-term remission after first-line therapy may still have detectable t(14;18) positive cells in circulation without any clinical evidence of active disease [1, 14]. In the course of disease, the disappearance or strong clearing of t(14;18) positive cells from the blood correlates with the clinical response to therapy [1].

The B-origin of tumor cells was demonstrated by the IHC positivity for L26 (CD20), which was recorded in all tested cases. In grade 1 FL, small lymphocytes were difficult to differentiate form tumoral cells.

CD45RO (UCHL1) was useful in identifying reactive small peri-follicular or intra-follicular lymphocytes.

We found bcl-2 (76.6%) and CD10 (71.5%) positive IHC expressions in most of our cases, similar with previously reported data in literature [15]. Bcl-2 is usually down-regulated in the normal follicle center, but is expressed in neoplastic follicle centre cells. However, approximately 10% of FL reported worldwide do not express bcl-2 [16]. The relatively high percentage (23.4%) of bcl-2 negative cases is different from other reports [16]. In 40% of the bcl-2 protein positive cases, bcl-6 was also positive. Bcl-2 is a phenotypic marker useful in differentiating FL from reactive follicular hyperplasia. All three cases (reactive proliferations) excluded from the initial group presented no IHC reaction for bcl-2. Bcl-2 protein and CD10 are considered reliable markers in diagnosing germinal centre B-cell lymphoma, in particular FL [16].

Immunohistochemical staining for bcl-2 and CD10 were predictive for the presence of the t(14;18) translocation, since 66.6% of the cases with t(14;18)

presented positive immunohistochemical reaction for bcl-2, and 35.7% for CD10. Although the study group included bcl-2 protein positive/t(14;18) negative cases (17.64%), the statistically significant positive correlation between the IHC expression for bcl-2 and the t(14;18) translocation detected by FISH (p=0.04), indicates that the IHC testing for bcl-2 protein in our series was reliable in predicting t(14;18) positive cases. This result has to be interpreted taking into consideration the limited number of cases. The high number of t(14;18) positive FL grade 3 obtained in our study may be explained by the predominance of this grade in the study group randomly selected for FISH.

The absence of bcl-2 positivity in IHC or FISH must not be taken as an assurance of benignity; in these cases, the distinction between FL and reactive hyperplasia should be made on morphological features.

FISH testing allows the identification of genetic abnormalities in follicular lymphomas and even a retrospective cytogenetic diagnosis on old paraffin-embedded specimens.

Conclusions

Immunohistochemical staining for bcl-2 and CD10 are useful predictive markers for follicular lymphomas.

Bcl-2 t(14;18) translocation plays an important role in the pathogenesis of follicular lymphomas, and is an important tool (FISH) in the diagnosis, treatment and follow up of these malignancies, since the immunohistochemical testing is negative in a significant proportion of cases.

Acknowledgements

This study was supported by the National Research Program PNCD No. 41–093/2007.

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Corresponding author

Alina Georgescu, MD, PhD, Department of Pathology, "Victor Babeş" National Institute of Pathology, 99–101 Independenței Avenue, 5th Sector, 050096 Bucharest, Romania; Phone +40723–432 134, e-mail: georgescu.alina@gmail.com

Received: August 20th, 2010 *Accepted: October 30th, 2010*