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Detection of t(14;18) by PCR of IgH/BCL2 Fusion Gene in Follicular Lymphoma from Archived Cytological Smears

Tajana Štoos-Veić[1], Ana Livun[2], Radmila Ajduković[3], Vlatko Pejša[3,4], Ozren Jakšić[3,4] and Rajko Kušec[2,4]

[1] Department of Cytology and Cytometry, Dubrava University Hospital, Zagreb, Croatia
[2] Department for Molecular Diagnostics and Genetics, Dubrava University Hospital, Zagreb, Croatia
[3] Department of Hematology, Dubrava University Hospital, Zagreb, Croatia
[4] Zagreb University, School of Medicine, Zagreb, Croatia
[5] University of Applied Health Studies, Zagreb, Croatia

A B S T R A C T

According to WHO classification follicular lymphoma (FL) is a neoplasm composed of follicle centre (germinal centre) B-cells, which usually has at least a partially follicular pattern. Bone marrow (BM) infiltration by lymphoma occurs in 40-70% of cases at the time of diagnosis. The characteristic chromosomal translocation of follicular lymphoma is t(14;18)(q32;q21) with transposition of BCL2 oncogene to the regulatory region of immunoglobulin heavy chain gene IgH. Aim of this study was to determine the frequency of PCR detection of IgH/BCL2 in DNA samples isolated from archival cytological slides of lymph node aspirates, bone marrow and/or peripheral blood (PB) obtained from patients with histologicaly confirmed follicular lymphoma using primers and protocol proposed by BIOMED-2 consortium. We also compared molecular with cytomorphological findings in bone marrow/peripheral blood and tested this method of detection of IgH/BCL2 molecular marker in monitoring minimal residual disease (MRD) in routine clinical setting. DNA was successfully isolated from all archival cytological slides obtained by fine needle aspiration of lymph nodes as well as from 75% of smears of bone marrow aspirates from 19 patients. Fusion oncogene was detected in 10 of 19 patients (52%). For patients with PCR IgH/BCL2 positive lymph nodes, molecular test found BM infiltration in 5 cases (83%), while cytomorphology detected infiltration in three of eight cases (37%) available for comparison. May-Grünwald-Giemsa stained cytological smears can be used for PCR-based ancillary methods and the rate of detection of IgH/BCL2 rearrangement is similar to results reported for paraffin-embedded tissues. For patients with detectable baseline molecular marker, PCR is a highly suitable method for detection of bone marrow involvement and monitoring MRD.

Key words: *follicular lymphoma, t(14;18), BCL2, PCR, minimal residual disease*

Introduction

According to WHO classification follicular lymphoma (FL) is a neoplasm composed of follicle centre (germinal centre) B-cells, which usually has at least a partially follicular pattern[1]. These neoplasms account for almost 30% of all non-Hodgkin lymphomas, and vast majority (80–90%) are low-grade lymphomas (grade 1–2), which, by consensus, should be separated from grade 3 lymphomas. FL typically affects lymph nodes, but spleen, bone marrow and peripheral blood are also frequently involved. Bone marrow (BM) infiltration by lymphoma occurs in 40–70% of cases at the time of diagnosis, with only 1/3 of the patients being in stage I or II at that time. The characteristic chromosomal translocation of follicular lymphoma is t(14;18)(q32;q21) with transposition of BCL2 oncogene to the regulatory region of immunoglobulin heavy chain gene IgH. It is present in approximately 90% of grade 1–2 FL, but it is much less frequent in grade 3 follicular lymphomas[2]. Reported frequencies depend on the technique used for detection[3,4]. Polymerase chain reaction (PCR) is a sensitive molecular test but detection of IgH/BCL2 fusion gene by PCR can fail due to the genomic variability of the breakpoints in BCL2 gene

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on chromosome 18[5]. Approximately 60% of BCL2 breakpoints are located in major breakpoint region (MBR) and 5–25% in minor cluster region (mcr), but other breakpoints have been found. Taking into account this inherent relative weakness of molecular designs for the variable genomic structure of the target oncogene, significant effort has been put in designing optimal molecular PCR based test for IgH/BCL2 detection. BIOMED-2 PCR protocol is recommended for t(14;18) detection and if positive in primary tumour, it becomes a valuable tool for precise monitoring of patient's disease[6].

Aim of this study was to determine the frequency of detection of t(14;18) by consensus BIOMED-2 PCR assay for IgH/BCL2 fusion gene on DNA isolated from archived cytological slides of primary tumours (lymph nodes), bone marrow and/or peripheral blood (PB) obtained from patients with histologicaly confirmed follicular lymphoma. We also sought to compare molecular with cytomorphological findings in bone marrow and/or peripheral blood of FL patients and we tested the value of this method of detection of IgH/BCL2 molecular marker in monitoring minimal residual disease (MRD) in BM and/or PB compartment after immuno and cytotoxic therapy.

Materials and Methods

Cytological smears of lymph node aspirates from 19 patients with histologicaly confirmed diagnoses of follicular lymphoma were randomly selected from our archive and reviewed for diagnosis and celularity. Clinical data for these patients are shown in Table 1. Clinical response criteria were defined according to revised response criteria for malignant lymphoma[7]. Specimens were obtained by fine needle aspiration (FNA) for diagnostic purposes[8] and slides were stored for 2–10 years. Two to four air-dried May-Grünwald Giemsa stained slides per case were selected for DNA extraction. DNA was isolated by previously described method according to standard phenol/chloroform procedure[9,10]. Patients with detected baseline molecular marker, had molecular monitoring during the follow up period performed on either fresh BM and/or PB cells or from archived cytological slides of those samples**,** when available (Table 2). PCR detection of the IgH/BCL2 rearrangement with amplification of the MBR, mcr and 3'MBR breakpoint regions was performed using the primers and protocol proposed by BIOMED-2 consortium[6]. Special attention was paid to prove that quality of DNA obtained from archived glass slides of cytological preparations was adequate for PCR and this was done by amplification of Abl as the control gene.

Results

Clinical characteristics of patients with FL, including histological grade, clinical staging and results of BIOMED-2 PCR performed on DNA obtained from lymph nodes are presented in Table 1. There were 15 females and four male patients, aged 25–73 years (median 53 years). Fifteen patients had low-grade (grade 1 and 2) lymphoma while only 4 patients were classified as follicular lymphoma grade 3. At the time of diagnosis, 2/3 of the patients were in clinical stage (CS) III and IV. Abl housekeeping gene amplification was successful in all samples of DNA isolated from archival cytological slides obtained by FNA of lymph nodes of 19 patients with FL at the time of diagnosis. BIOMED-2 IgH/BCL2 protocol has detected fusion oncogene in 10 of 19 patients (52%). Eight PCR-positive patients (80%) had low-grade FL. Two of four (50%) grade 3 FL patients were IgH/BCL2 positive (Table 1). Except for one, all patients exhibited BCL2 MBR type breakpoint. For eight patients with PCR IgH/BCL2 positive lymph nodes the same molecular test was performed on DNA isolated from cytological slides of BM aspirates (and PB when available) at the time of diagnosis and some time after therapy (Table 2). Example of agarose gel electrophoresis results is shown in the Figure 1. Two of analyzed BM samples (25 %) were considered technically inadequate for molecular analysis (No 10 and 14), as there was no amplification of the control gene. Molecular test found BM infiltration in 5 out of six cases (83%) with good quality DNA, while cytomorphology detected infiltration in three of eight examined cases (37%) at the time of diagnosis (Table 2). Of the two patients with PCR-detectable molecular marker in BM at initial staging and who were available for molecular follow up, one (Number 1) remained positive after immunocytotherapy although in clinical remission (CR) and with

TABLE 1
CLINICAL AND MOLECULAR CHARACTERISTICS OF FOLLICULAR LYMPHOMA PATIENTS

Patient No	Age	Gender	Grade	Clinical Stage	Lypmh nodes PCR
1	56	F	1	IIA/B	+
2	42	F	2	IIA	+
3	48	F	2	IIIA	+
4	37	F	1	IVA	–
5	67	F	3	IIIA	–
6	25	M	3	IIA	+
7	52	F	1	IVA/B	–
8	48	F	1–2	IVA	–
9	57	M	1	IVA/B	–
10	67	F	1	IVA/B	+
11	46	F	1	IVB	+
12	57	M	1	IVA/B	–
13	51	F	3	IVA/B	–
14	71	F	1	IVA/B	+
15	52	F	1	IIA	–
16	48	F	3	IIA	+
17	58	M	1	IVA/B	–
18	52	F	1	IIA/B	+
19	73	F	1	IVA	+

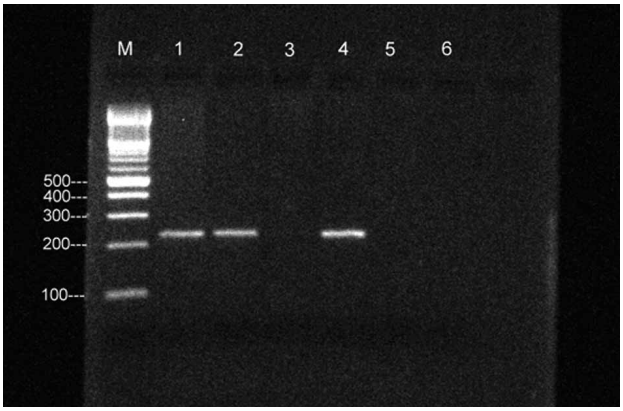

Fig. 1. Representative agarose gel electrophoresis of IgH-BCL2 PCR amplicons M-molecular weight marker 100bp-allelic ladder, 1,2-lymph node (smear sample), 3-bone marrow, weak positive (smear sample), 4-lymph node (fresh FNA aspirate), 5-peripheral blood, 6-negative control, water blank.

clearing of the infiltrate as detected by cytomorphology. The second patient (Number 18) became PCR-negative after therapy. Cytomorphology failed to detect infiltration of BM for this patient both at initial staging and after therapy (Table 2). A single patient with PCR and cytomorphologicaly negative bone marrow (Number 2) remained negative and is in prolonged complete remission. One patient with stage IVB (Number 14) had aggressive disease and was treated with several lines of immuno (MabThera®) and cytotoxic therapies for resistant disease. She was both PCR and morphologically constantly positive in BM and PB and had succumbed to the disease. Summarized, PCR is superior to cytomorphology in detecting lymphoma infiltration of BM at the time of diagnosis. This advantage is retained or is even more evident in evaluating the same compartment after immuno or cytotoxic therapy of FL.

Discussion and Conclusion

Molecular markers in haematologic malignancies, when present, are used for establishing diagnosis, contribute to process of clinical staging (evaluation of involvement of BM or PB), serve as prognostic factors and are useful for detection of minimal residual disease. Detection of t(14;18) is not required for establishing diagnosis of FL, but may prove important for confirmation of diagnosis when standard analyses are inconclusive. FISH and PCR are molecular cytogenetic methods regularly used for detection of t(14;18)(q32;q21) with formation of the fusion oncogene IgH/BCL2 in follicular lymphomas. Although PCR is the most sensitive and specific molecular test for detection of genetic aberrations occurring through chromosome rearrangements, in FL it detects the specific genetic marker in only 50–70% of cases at diagnosis[11,12]. On the other hand, interphase FISH is more sensitive because it can detect breakpoints occurring outside regions covered by PCR and confirms t(14;18) in 90% FLs harbouring this rearrangement in its karyotype[13,14]. While this fact is of advantage at the

TABLE 2
MOLECULAR AND CYTOMORPHOLOGICAL MONITORING OF IGH/BCL2 POSITIVE PATIENTS (BONE MARROW AND PERIPHERAL BLOOD)

Patient No	At diagnosis		After therapy at			Current status
	PCR	Cytology	PCR	Cytology		
1	■	■	■	□	+1m	CR
	○	○		○		
2	□	□	□	□	+24m	CR
		○	○	○		
6	■	□		□	+1m	CR
		○	○	○	+24m	
10	*	■	■	□	+8m	PR
		●	○	○	+24m	
11	■	□		□	+2m	CR
	●	○		○		
14	*	■	■	■	+1m	died at +2m
		●	●	●		
18	■	□	□	□	+1m	CR
		○	○	○		
19	■	□		□	+12m	PR
		○	○	○	+12m	

● PB – peripheral blood positive, ○ PB – peripheral blood negative, ■ BM – bone marrow positive, □ BM – bone marrow negative, CR – complete response, PR – partial response, * – no amplification of the control gene, +m – month after therapy

time of diagnosis, it is less useful for monitoring the disease throughout the treatment. If PCR can detect IgH/BCL2 in primary tumour, high sensitivity and specificity make this method a valuable tool for monitoring patients with FL and detection of MRD[15]. It is also well known that paraffin-embedded tissue samples are reliable source of DNA for molecular analysis[16–18]. In our study we have shown that air-dried, routinely stained and stored cytological specimens can also provide material for molecular analysis as 100% of lymph node material and 75% of bone marrow specimens had good quality DNA preserved (Table 1 and 2). The length of time slides have been stored didn't have impact on DNA amplification. For two samples of the bone marrow specimens that didn't amplify housekeeping gene the reason for failure is not clear. Unexpected advantage of the method was that even slides that were less satisfactory for morphology and not suitable for other ancillary techniques such as immunocytochemistry due to large numbers of naked nuclei, were a good source of DNA. Cytological slides have already been evaluated for DNA extraction[7,9], but to our knowledge detection of IgH/BCL2 translocation was not examined. Espinet observed IgH/BCL2 translocation in 59% of FL using PCR BIOMED-2 protocol on DNA isolated from paraffin-embedded tissue, and Belaud-Rotureau had similar results on frozen and paraffin-embedded tissue[11,12]. In our study frequency of detection of IgH/BCL2 in lymph nodes was 52%, and of 15 low-grade follicular lymphomas 53% were positive, in keeping with reported results.

According to WHO classification, bone marrow infiltration occurs in 40–70% of FL cases at presentation, but depending on the method used for evaluation, bone marrow is infiltrated with lymphoma cells in majority of cases of follicular lymphoma at initial staging even if there is no evidence of involvement based on morphological evaluation only. Most cases of indolent lymphoma eventually relapse despite treatments, probably because of persistence of malignant cells that are undetectable by conventional methods. PCR-based methods are increasingly used for minimal residual disease detection, as they have higher sensitivity compared to morphological and immunophenotyping techniques[19]. Several studies showed that molecular response after conventional chemotherapy with or without rituximab (antiCD20Ab) was favourable prognostic factor[20,21]. In our series of available patients, molecular test detected MRD where cytomorphology did not have proof of persisting neoplastic cells. BM clearance was found by PCR in one case, three cases remained positive and three were not available for monitoring of MRD in bone marrow. Of three cases positive after therapy, two are in clinical remission and one patient had succumbed to the disease.

In conclusion, we can state that MGG stained cytological smears can be useful as a source of DNA for PCR-based ancillary methods especially in cases where amount and quality of diagnostic material is limited. Frequency of detection of IgH/BCL2 rearrangement is similar to results reported for paraffin-embedded tissues. Although on limited number of cases, we have confirmed that current consensus IgH/BCL2 BIOMED-2 PCR test is not optimal method for the detection of translocation t(14;18) in follicular lymphoma, but for PCR-positive cases it is a highly suitable method for detection of bone marrow involvement and monitoring MRD.

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T. Štoos-Veić

Department of Cytology and Flow Cytometry, Dubrava University Hospital, Av. Gojka Šuška 6, 10000 Zagreb, Croatia
e-mail: tveic@kbd.hr

PCR ZA OTKRIVANJE IGH/BCL2 PREUREDBE S ARHIVSKIH CITOLOŠKIH RAZMAZA PUNKTATA FOLIKULARNIH LIMFOMA

S A Ž E T A K

Prema klasifikaciji Svjetske zdravstvene organizacije, folikularni limfomi su tumori stanica centra folikula koji bar djelomice zadržavaju folikularni način rasta. Infiltracija koštane srži nalazi se u 40–70% slučajeva u vrijeme postavljanja dijagnoze. Karakteristična kromosomska translokacija folikularnih limfoma je t(14;18)(q32;q21) koja premješta BCL2 onkogen pod kontrolu promotorske regije za teške lance imunoglobulina IgH na kromosomu 14. Cilj ovog rada bio je odrediti učestalost translokacije t(14;18) molekularnom analizom temeljenom na polimeraza lančanoj reakciji (PCR) u uzorcima DNA izoliranim s arhivskih citoloških stakala koristeći standardizirani analitički protokol Europske radne grupe BIOMED-2, usporediti molekularne i citomorfološke nalaze te ispitati vrijednost metode za praćenje minimalne ostatne bolesti (MRD) u svakodnevnim uvjetima. Izolacija DNA je uspjela sa svih citoloških razmaza punktata limfnih čvorova kao i u 75% razmaza punktata koštane srži, a PCR-om je otkrivena IgH/BCL2 preuredba u 10 od 19 (52%) bolesnika. U bolesnika s pozitivnim molekularnim testom u limfnom čvoru, PCR-om je pronađena infiltracija koštane srži limfomom u 5 bolesnika (83%), dok je citološka analiza infiltraciju našla u 3 od osam uzoraka dostupnih za usporedbu. Citološki razmazi bojani May-Grünwald Giemsa metodom (MGG) dobar su izvor DNA za metode molekularne analize temeljene na lančanoj reakciji polimeraze. Učestalost otkrivanja IgH/BCL2 preuredbe odgovara rezultatima studija rađenih na histološkim uzorcima. U bolesnika u kojih je pronađena translokacija u dijagnostičkim uzorcima, PCR je pogodna metoda za otkrivanje infiltracije koštane srži limfomom te za praćenje minimalne ostatne bolesti.