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Evaluation of t(14;18)(q32;q21) and BCL2 protein and their prognostic role in follicular lymphoma

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- t(14;18)(q32;q21)
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- Follicular lymphoma
- MBR
- icr
- mcr

Abstract

Background: Previous reports have suggested the significant association of t(14;18)(q32;q21) and follicular lymphoma (FL). However, little information is available in the literature on the relationship between BCL2 protein, BCL2 gene status in FL and the patient outcomes. Also, understanding of IGH/BCL2 molecular rearrangement using real time PCR (RT-PCR), in follicular lymphoma in relation to survival might provide a more accurate and rational method of risk stratification to guide treatment and might suggest new therapeutic approaches as well. **Methods**: This study evaluated the relative frequency of t(14;18) by RT-PCR and its apoptosis-related BCL2 protein expression by an immunohistochemical assay (IHC) in fifty FL cases in tissues. In addition, we evaluated the relation of BCL2 protein expression to the translocation, together with the relation of both t(14;18) and BCL2 protein expression to the clinico-pathological features and survival data including progression free survival (PFS); and overall survival (OS) in order to evaluate their prognostic role in FL. **Results & conclusion**: There was a significant association of the t(14;18) with BCL2 protein expression, grading of FL, and the OS. In addition, there was a significant association of BCL2 protein expression with the grading of FL, OS, International Prognostic Index (IPI) score and performance status. However no significant association of t(14;18) or BCL2 protein expression with the other clinico-pathological features, and PFS.

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INTRODUCTION

FL; a subtype of non-Hodgkin's lymphoma (NHL), constitute 20%–30% of all lymphoid tumors with the highest incidence in Western countries [1,2]. However, in other countries the relative incidence is much lower. In Egypt, it represents about 5.2% at the National Cancer Institute Registry [3]. FL affects predominantly older adults. Most patients possess a widespread disease at diagnosis [4].

The chromosomal translocation t(14;18) (q32;q21)–IGH/BCL2 (immunoglobulin heavy chain/B-cell leukemia/lymphoma) occurs in 70% to 95% of all FL cases. Its molecular consequence is the juxtaposition of the BCL2 protooncogene (18q21) with the enhancer sequences of the IGH gene promoter region (14q32), resulting in an overexpression of the BCL2 oncogene in the neoplastic follicles [2,5].

The BCL2 protooncogene, which is a potent anti-apoptotic molecule, is expressed normally in resting B cells present in the perifollicular mantle zone and in post-follicular B cells. Germinal center (GC) B cells, however, physiologically lack BCL2 protein expression and undergo apoptosis unless they are selected by specific antigens that drive them into somatic hypermutation and class switching. Due to the lack of BCL2 expression, among other factors, the large bulk of B-cells that enter the GC microenvironment will be removed by apoptosis. The constitutive overexpression of BCL2 in GC B cells resulting from the t(14;18)(q32;q21) leads to an accumulation of inappropriately rescued B cells with a prolonged life span, allowing for the development of additional genetic hits, that are needed for the establishment of overt FL. Variant translocations of the t(14;18) as the t(2;18) or t(18;22), juxtapose BCL2 to the loci of the immunoglobulin light chains (κ, λ) and in like manner, resulting in inappropriate and sustained BCL2 expression in GC B cells [2].

In lymphoma, BCL2 protein inhibits apoptosis and cell death, which is a fundamental step of malignant transformation, leading to apoptosis suppression and progression to lymphoma. BCL2 protein overexpression is an essential factor related to multiple drug resistance and absence of response to chemotherapy [6]. The presence of the t(14;18) itself is not sufficient to bring about neoplastic transformation of B cells, as BCL2 transgenic mice develop lymphomas only after long latency periods and after secondary chromosomal alterations [7].

Sixty to seventy % of breakpoints in the BCL2 gene on chromosome 18 occur in the major breakpoint region (MBR) located in 3' untranslated (non coding) region of the exon 3 of this gene and 3% to 30% occur in the minor cluster region (mcr) located about 20 kb distally from MBR in the 3' flanking region, and the rest are widely scattered over the BCL2 genomic region. One of them is the intermediate cluster region (icr), located approximately 19 kb downstream of the MBR. It accounts for about 10% of t(14;18) incidence in FL. The breakpoints on chromosome 14 mostly lie in the joining region (J) on IGH [1,8].

Identification of IgH/BCL2 fusion in FL is clinically essential for building up a diagnosis and observing disease progression [9,10]. This study aimed at evaluation of the relative frequency of

t(14;18) using RT-PCR and BCL2 protein expression by IHC in fifty FL cases in tissues. In addition, their correlation with each other and with the clinico-pathological features and survival data including PFS and OS in order to evaluate their prognostic role in FL.

MATERIAL AND METHODS

1. Patients

This work was done on 50 diagnosed cases of FL underwent excisional lymph node biopsy. All cases were received in the pathology laboratory of Mansoura Oncology center, Mansoura University in the period between January 2014 and January 2016. The medical records of patients were reviewed to assess the clinical data including (age, sex, performance, International Prognostic Index (IPI) score, Lactate dehydrogenase (LDH) serum level, Staging, extranodal involvement, bone marrow (B.M) and central nervous system (C.N.S) involvement, anemia, B symptoms, previous treatment, clinical relapse, clinical response to therapy and survival data including PFS and OS.

2. Microscopic and immunohistochemical staining:

Haematoxylin and Eosin stain (H&E) slides were reviewed to confirm the diagnosis based on morphologic defining criteria of WHO 2008 classification of lymphoma [11]. Immunohistochemical panel was used to confirm the diagnosis, this panel included (LCA, CD20, CD3, CD10, CD5, cyclin D1 and BCL6). BCL2 protein expression was assessed for all cases using (Mouse monoclonal anti BCL2. CELL MARQUE, code No 226M-98, 7 ml prediluted, and Rabbit monoclonal anti Ki67 (Clone: SP6). GENNOVA, code No AP10244, 7 ml prediluted), (Invitrogen; Histostain –SP broad spectrum (HRP). Catalog No 959943B) as secondary antibody system and (DAB substrate kit, CELL MARQUE, catalog No 957D-30) as a detection system. The positive control, a normal lymph node was used (BCL2 is normally expressed in Mantle zone of lymphoid follicle); for the negative control, the primary antibody was omitted. Procedure of immunostaining was according to Taylor [12].

Interpretation of immunohistochemical staining

The BCL2 positivity was determined by cytoplasmic staining (brown) of neoplastic cells. The internal control was T cells. The percentage of positive cells at the whole section after exclusion of the areas of reactive T cells was determined. It was scored negative if 5% or less of neoplastic cells were stained. In this study, the positive cases were scored from 6% to 100%. The value of BCL2 was considered weak positive if 6 to less than 50% were brown stained and strong positive if $\geq$ 50% of tumor cells were brown stained [13].

3. RT-PCR for detecting BCL2 rearrangements

Extracted DNA from paraffin-embedded tissue samples was prepared, using G-spin™ total DNA extraction kit supplied by Intron, Korea, according to the manufacturer's instructions. Nano Drop 2000 spectrophotometer (ThermoFisher scientific, USA) was used to assess purity and concentration of the extracted DNA.

Extracted DNA samples were tested for IGH/BCL2 molecular rearrangement using RT-PCR protocol that used specific primers for MBR, mcr, icr and JH consensus regions. ß- globin gene was used as a housekeeping gene. The primer sequences used were as follows: MBR: 5'-TTA GAG AGT TGC TTT ACG TG-3' forward [14], mcr: 5'-GAC TCC TTT ACG TGC TGG TAC C-3' forward [15], icr: 5'-TGC AGA ATC TGA CGT TCA GTC A-3' forward [16] and JH consensus: 5'-ACC TGA GGA GAC GGT GAC-3' as common reverse [17]. The forward primer of ß-globin was 5'-GTA CGG CTG TCA TCA CTT AGA C-3' and the reverse: 5'-AAA CCC AAG AGT CTT CTC TGT C-3' [18].

PCR amplifications were performed using Maxima SYBR Green qPCR master mix kit supplied by ThermoFisher scientific, USA. PCR reactions was performed separately for each of MBR, mcr, icr and ß-globin. The reaction mixture of 20 µL contained 50 ng of DNA, 0.6 µL of the specific forward primer for each breakpoint or ß-globin, 0.6 µL of the reverse primer (JH consensus or ß-globin reverse), 10 µL of the master mix and nuclease free water up to 20 µL. The optimized thermal profile included initial denaturation at 95°C for 15 min. followed by 40 cycles of 95°C for 15 sec. and 60°C for 60 sec. Melting curve analysis was performed to assess the specificity of the amplification product. PCR amplification was done using Rotor gene Q (Qiagen, Germany).

4. Statistical analysis

Statistical analysis was done using SPSS 18.0 (SPSS, Chicago, IL, USA). Mean ± standard deviation (SD) was used to describe Quantitative variables, while number and percentage were used to describe categorical variables. The chi square test or exact fisher test and Mann-Whitney U test or analysis of variance (ANOVA) test were used to compare translocation positive and translocation negative groups and BCL2 positive and negative cases, according to the clinicopathological variables, OS and PFS. A linear regression analysis was used for evaluation of the significance of the independent associations between the covariates and PFS and/or OS. All statistical tests were two sided and significance was defined as $p < 0.05$.

RESULTS

Clinicopathological features of studied cases

Fifty patients of FL, admitted to the Oncology centre, Mansoura University for treatment were studied retrospectively. The clinicopathologic features are summarized in table (1). The age of the patients ranged between 27-83 years with a mean age of 59.8 ±11.5 years. Out of the 50 patients, 36 (72%) were males and 14(28%) were females. The study revealed that 46 patients (92%) of the studied FL cases initially presented with level 1 of performance Status and 47 (94%) of patients had high blood level of LDH (more than 450U/L). The IPI score, which identifies subgroups of patients with a very poor or a good outcome [19], was low (1&2) in 24 (48%) of NHL patients. As regarding B symptoms, 37 cases (74%) were initially

Table (1): Clinicopathological features of follicular lymphoma cases.

Variable	Number	Percentage (%)	Variable	Number	Percentage (%)
Age			**IPI score**		
>60y	17	34	*1&2*	24	48
≤60y	33	66	*3&4*	26	52
Gender			**Anemia**		
Male	36	72	*Present*	18	36
Female	14	28	*Absent*	32	64
Staging			**Serum LDH**		
Stage1&2	37	74	*Normal*	3	6
Stage3&4	13	26	*High*	47	94
Extranodal involvement			**B symptoms**		
Positive extranodal sites	13	26	*Present*	13	26
No extranodal sites	37	74	*Absent*	37	74
Grading			**BM/CNS involvement**		
GradeI	9	18	*Present*	11	22
Grade II	20	40	*Absent*	39	78
Grade IIIA	15	30	**Clinical response to therapy**		
Grade IIIB	6	12	*Complete response*	9	18
			No complete response	41	82
Performance levels			**Clinical relapse**		
Level 1	46	92	*Present*	10	20
Level 2	4	8	*Absent*	40	80

presented without B symptoms, only 13 cases (26%) presented with B symptoms. No one of patients presented by bulky disease. 18 cases (36%) presented with anemia. The mean follow up time was 35.8 ± 8.5 months; cases with lost follow up were excluded from this study.

Regarding staging of FL, 13 cases (26%) presented at advanced stage (3&4) while 37 cases (74%) were in early stage (1&2) at presentation. Extranodal involvement was present in 13 (26 %) cases. These extranodal sites were paraspinal, chest wall and oropharynx. Histopathologically, the lymphoma cells were mixture of small centerocytes and larger centeroblasts in various proportions in different areas. The pattern of arrangement was follicular in 22 cases, 12 were of diffuse pattern and 16 cases were of mixed follicular and diffuse pattern. Regarding grading of the included cases, 9 cases (18%) were grade I, 20 cases were grade II (40%) and 21 cases (42%) were grade III (GIIIA, GIIIB).

All cases were treated with chemotherapy without rituximab. Regarding clinical response to therapy in the 50 FL cases, complete response occurred in 9 (18%) cases. However remaining cases (82%) showed no complete response to therapy (progression in 10 (20%) patients cases, stationary course in 12 (24%) patients, partial remission in 11 (22%) and 8 patients (16%) died at the follow up period (3%) patients).

Regarding clinical relapse, 10 patients (20%) have relapsed disease within the follow up period;

7 (14%) after complete remission and 3 cases (6%) after partial remission. On the other hand, 40 patients (80%) did not show relapse.

Survival analysis and its relation to the clinicopathologic parameters

In our study population, the PFS of 50 patients ranged from 0-59 months with mean±SD 23.12±18.95 months for all cases. No significant correlation between PFS and other clinico-pathologic parameters and response to therapy apart from B symptoms (p value=0.004). On the other hand, the OS ranged from 1-59 months with mean±SD 33.76± 18.65 months and with no statistically detected significance to other clinicopathologic features.

Frequency of translocation and different breakpoints

The translocation was detected in 43 FL cases (86%) and 7 cases (14%) showed no translocation. The distribution of BCL2 breakpoints was as follows: MBR in 32 patients (64%), icr in 8 patients (16%) and mcr in 3 patients (6%). The remaining 7 cases (14%) were negative for any breakpoints by all primers used in this study.

Frequency of BCL2 protein expression

BCL2 was positive in 41 cases (82%) of FL and negative in 9 cases only (18%). However, the expression of BCL2 was not always homogeneous and demonstrated a variable degree of heterogeneity; among the BCL2 positive lymphoma cases, 38 were strong positive (partial or diffuse) and the remaining 3 cases were weak (Figure 1).

Comparing between translocation positive and negative FL cases and between different breakpoints regarding clinicopathologic and survival data and BCL2 protein expression

The differences between translocation positive and negative FL cases and between the FL cases with different breakpoints regarding clinico-pathologic and survival data, and BCL2 protein expression were summarized in tables (2,3).

There was highly significant association between histological grade and the presence of translocation (p value<0.001). **96.6%** (28/29) of low grade (GI&GII) FL and **71.4%** (15/21) of high-grade (GIIIA, GIIIB) FL were translocation positive cases. On the other hand, **3.4%** (1/29) of low grade (GI&GII) FL and **28.6%** (6/21) of high-grade (GIIIA, GIIIB) FL were translocation negative FL cases. This indicates that the translocation was more frequent in low grade FL and the absence of translocation was more frequent in high grade FL. The least frequency of translocation was in grade IIIB and translocation negative FL cases had the highest frequency in grade IIIB. In addition, the different breakpoints were statistically higher (p=0.019) in those with low-grade lymphoma (20/29; **68.9%** for MBR, 5/29; **17.2%** for icr, 3/29; **10.3%** for mcr) than in those with high-grade lymphoma (12/21; **57.1%** for MBR, 3/21; **14.3%** for icr, 0/21; **0%** for mcr). Moreover, MBR had the least frequency in grade IIIB, icr was absent in both grades I and IIIB, and mcr was absent in both grades IIIA and IIIB. On the other hand, no significant association between translocation and age, gender, staging, extranodal involvement, CNS or BM involvement, performance status, IPI score, LDH, B symptoms, clinical response to therapy and relapse.

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Table (2) Relation of translocation and breakpoint site with clinicopathologic and survival data, and BCL2 protein expression in the studied 50 follicular lymphoma cases.

t(14;18) / Variable	Positive t(14;18) N (%) = 43 (86%)			Negative t(14;18) N (%) = 7 (14%)	P value
	MBR 32(64%)	icr 8(16%)	mcr 3(6%)		
Qualitative variables N (%):					
• **Gender**					
Male	25(78.1)	4(50.0)	3(100)	4(57.1)	0.23
Female	7(21.9)	4(50.0)	0(0.0)	3(42.9)	
• **Staging**					
Stage 1&2	24(75.0)	6(75.0)	2(66.7)	5(71.4)	1.0
Stage 3&4	8(25.0)	2(25.0)	1(33.3)	2(28.6)	
• **Extra nodal involvement**					
Yes	9(28.1)	1(12.5)	0(0.0)	3(42.9)	0.50
No	23(71.9)	7(87.5)	3(100)	4(57.1)	
• **Grade #**					
FL GI	8(88.9)	0(0.0)	0(0.0)	1(11.1)	
FL GII	12(60)	5(25)	3(15)	0(0.0)	**<0.001***
FL GIIIA	11(73.3)	3(20)	0(0.0)	1(6.7)	
FL GIIIB	1(16.7)	0(0.0)	0(0.0)	5(83.3)	
• **Performance status**					
1	28(87.5)	8(100)	3(100)	7(100)	0.83
2	4(12.5)	0(0.0)	0(0.0)	0(0.0)	
• **IPI score**					
1	2(6.2)	0(0.0)	0(0.0)	0(0.0)	
2	16(50.0)	4(50.0)	1(33.3)	1(14.3)	0.29
3	4(12.5)	1(12.5)	2(66.7)	1(14.3)	
4	10(31.2)	3(37.5)	0(0.0)	5(71.4)	
• **Anemia**					
Positive	9(28.1)	4(50.0)	0(0.0)	5(71.4)	0.09
Negative	23(71.9)	4(50.0)	3(100)	2(28.6)	
• **LDH**					
High	29(90.6)	8(100)	3(100)	7(100)	1.0
Normal	3(9.4)	0(0.0)	0(0.0)	0(0.0)	
• **B symptoms**					
Positive	7(21.9)	2(25.0)	0(0.0)	4(57.1)	0.24
Negative	25(78.1)	6(75.0)	3(100)	3(42.9)	
• **Bone marrow / CNS involvement**					
Positive	10(31.2)	0(0.0)	0(0.0)	1(14.3)	0.22
Negative	22(68.8)	8(100)	3(100)	6(85.7)	
• **BCL2 protein expression**					
Strong diffuse	17(53.1)	8(100)	3(100)	0(0.0)0	
Strong partial	8(25.0)	0(0.0)	0(0.0)	2(28.6)	**0.01***
Weak partial	3(9.4)	0(0.0)	0(0.0)	0(0.0)	
Negative	4(12.5)	0(0.0)	0(0.0)	5(71.4)	
• **Therapy response**					
Complete response (CR)	4(12.5)	1(12.5)	2(66.7)	2(28.6)	0.1
No complete response	28(87.5)	7(87.5)	1(33.3)	5(71.4)	
• **Relapse**					
Yes	7(21.9)	0(0.0)	1(33.3)	2(28.6)	0. 34
No	25(78.1)	8(100)	2(66.7)	5(71.4)	
Quantitative variables (mean ±SD):					
• **Age (Years)**	56.38± 12.95	63.38± 9.55	63.0± 3.46	57.0± 6.98	0.41
• **Progression free survival (PFS) (Months)**	21.44± 19.25	24.0± 19.12	20.67± 17.9	30.86± 19.95	0.70
• **Overall survival (OS) (Months)**	37.22± 17.82	30.38±16.32	37.0± 10.39	20.43± 23.73	0.14

*** P value is significant** **# Percentage calculated from the total number of each grade**

Table (3): Comparison between translocation positive and negative follicular lymphoma cases regarding clinicopathologic and survival data and BCL2 protein expression.

t(14;18) / Variable	Positive t(14;18) N (%) = 43 (86%)	Negative t(14;18) N (%) = 7 (14%)	P value
Qualitative variables N (%):			
• **Gender** *Male* *Female*	 32(74.4) 11(25.6)	 4(57.1) 3(42.9)	0.38
• **Staging** *Stage 1&2* *Stage 3&4*	 32(74.4) 11(25.6)	 5(71.4) 2(28.6)	1.00
• **Extra nodal involvement** *Yes* *No*	 10(23.3) 33(76.7)	 3(42.9) 4(57.1)	0.27
• **Grade #** *FL GI* *FL GII* *FL GIIIA* *FL GIIIB*	 6(66.7) 20(100) 14(93.3) 3(50)	 3(33.3) 0(0.0) 1(6.7) 3(50)	**0.002****
• **Performance status** *1* *2*	 39(90.7) 4(9.3)	 7(100) 0(0.0)	1.00
• **IPI score** *1* *2* *3* *4*	 2(4.7) 21(48.8) 7(16.3) 13(30.2)	 0(0.0) 1(14.3) 1(14.3) 5(71.4)	0.17
• **Anemia** *Positive* *Negative*	 13(30.2) 30(69.8)	 5(71.4) 2(28.6)	0.08
• **LDH** *High* *Normal*	 40(93.0) 3(7.0)	 7(100) 0(0.0)	1.0
• **B symptoms** *Positive* *Negative*	 9(20.9) 34(79.1)	 4(57.1) 3(42.9)	0.07
• **Bone marrow / CNS involvement** *Positive* *Negative*	 10(23.3) 33(76.7)	 1(14.3) 6(85.7)	1.00
• **Therapy response** *Complete response (CR)* *No complete response*	 7(16.3) 36(83.7)	 2(28.6) 5(71.4)	0.4
• **Relapse** *Yes* *No*	 8(18.6) 35(81.4)	 2(28.6) 5(71.4)	0.62
• **BCL2 protein expression** *positive* *Negative*	 39(90.7) 4(12.59.3)	 2(28.6) 5(71.4)	**0.001***
Quantitative variables (mean ±SD):			
• **Age (Years)**	58.14± 12.2	57.0± 6.98	0.81
• **Progression free survival (PFS) (Months)**	21.86± 18.73	30.86± 19.95	0.25
• **Overall survival (OS) (Months)**	35.93± 17.06	20.43± 23.73	**0.04***

*** P value is significant** **# Percentage calculated from the total number of each grade**

Table (4) Comparison between BCL2 positive and BCL2 negative follicular lymphoma cases regarding clinicopathologic and survival data.

BCL2 expression / Variable	Positive BCL2 N (%) = 41 (82%)	Negative BCL2 N (%) = 9 (18%)	P value
Qualitative variables N (%):			
• **Gender**			
Male	31(75.6)	5(55.6)	0.25
Female	10(24.4)	4(44.4)	
• **Staging**			
Stage 1&2	31(75.6)	6(66.7)	0.68
Stage 3&4	10(24.4)	3(33.3)	
• **Extra nodal involvement**			
Yes	11(26.8)	2(22.2)	1.0
No	30(73.2)	7(77.8)	
• **Grade #**			
FL GI	8(88.9)	1(11.1)	
FL GII	18(90)	2(10)	**<0.001***
FL GIIIA	13(86.7)	2(13.3)	
FL GIIIB	2(33.3)	4(66.7)	
• **Performance status**			
1	40(97.6)	6(66.7)	**0.016***
2	1(2.4)	3(33.3)	
• **IPI score**			
1	2(4.9)	0(0.0)	
2	22(53.7)	0(0.0)	
3	8(19.5)	0(0.0)	**0.001****
4	9(22.0)	9(100)	
• **Anemia**			
Positive	14(34.1)	4(44.4)	0.71
Negative	27(65.9)	5(55.6)	
• **LDH**			
High	38(92.7)	9(100)	1.0
Normal	3(7.3)	0(0.0)	
• **B symptoms**			
Positive	9(22.0)	4(44.4)	0.21
Negative	32(78.0)	5(55.6)	
• **Bone marrow / CNS involvement**			
Positive	10(24.4)	1(11.1)	0.66
Negative	31(75.6)	8(88.9)	
• **Therapy response**			
Complete response (CR)	8(19.5)	4(44.4)	0.19
No complete response	33(80.5)	5(55.6)	
• **Relapse**			
Yes	7(17.1)	3(33.3)	0.36
No	34(82.9)	6(66.7)	
Quantitative variables (mean ±SD):			
• **Age (Years)**	58.49± 12.4	55.67± 6.67	0.51
• **Progression free survival (PFS) (Months)**	22.07± 18.61	27.89± 20.93	0.41
• **Overall survival (OS) (Months)**	39.88± 13.36	5.89± 13.18	**0.001****

*** P value is significant** **# Percentage calculated from the total number of each grade**

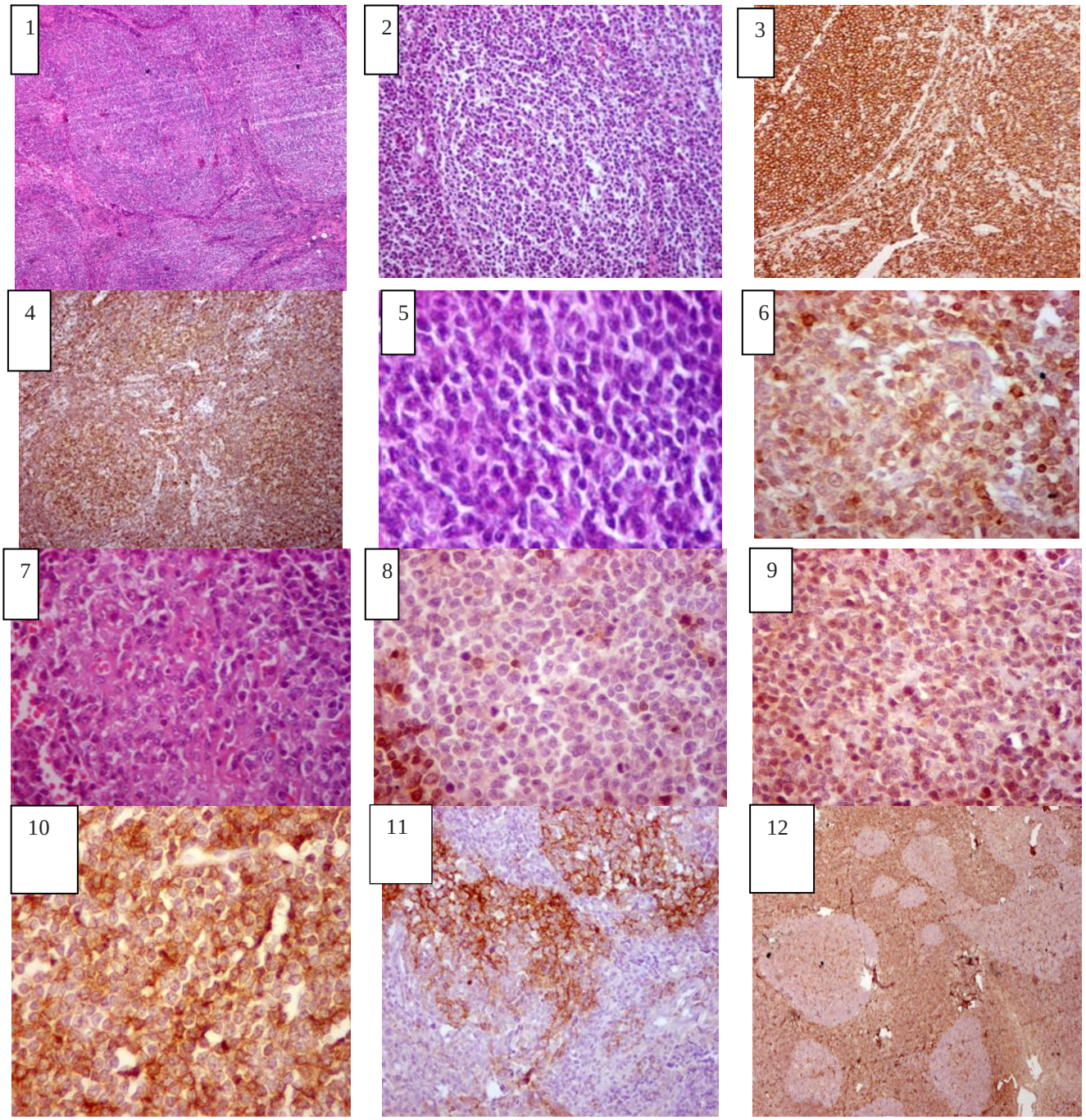

Figure (1):variable immunohistochemical expression of BCL2 in different grades of follicular lymphoma: Low grade ; G1 (photo 1x 40), and GII (photo 2x 200) follicular lymphoma exhibit follicular pattern formed mainly of small cleaved and non cleaved cells and showed diffuse membranous staining for CD10 (photo 3x 200) and strong cytoplasmic expression of BCL2 inside and outside the follicles (photo x4 200).G3A follicular lymphoma showed increased number of centroblasts (photo 5 x400) and showed moderate cytoplasmic expression of BCL2(photo 6 x400). G3B follicular lymphoma (photo 7 x400) showed negative cytoplasmic staining of neoplastic cells for BCL2 with positive internal control of T cells(photo 8x400) however it diffusely expressed CD20, Bcl6, (photo 9 x400(photo10x400 respectively)andfocal positivity for CD23. normal expression of BCL2 in reactive LNs (photo 12 x200) it showed positive staining in mantle zone with negative germinal centers

There was a significant association (p value= 0.04) between the OS and the presence of translocation. OS of translocation positive (mean ± SD=35.93± 17.06) was statistically favorable than OS of translocation negative FL (mean ± SD=20.43± 23.73). However, no significant association of translocation with PFS.

There was highly significant association between BCL2 expression and the presence of translocation (p value= 0.001), with 39/43 (90.7%) cases of t(14;18)-positive FL being positive for BCL2 with only 4/43 (9.3%) of the t(14;18)-positive FL showing negative BCL2 expression. Also, 2/7 cases (28.6%) of t(14;18)-negative FL were positive for BCL2, while 5/7 cases (71.4%) were BCL2 negative. In addition, there was a significant association between BCL2 expression and the different breakpoints, with the strong diffuse positive staining being the most frequent in those with positive MBR, the only finding in those with icr or mcr and absent in those without t(14;18). The strong partial, weak partial and negative staining for BCL2 was only observed in those with MBR and those without t(14;18).

Comparing between BCL2 positive and negative FL cases regarding clinicopathologic and survival data.

The differences between BCL2 positive and BCL2 negative FL cases regarding clinicopathologic and survival data and response to therapy were summarized in Table (4). There was statistically significant relation between BCL2 expression and pathologic grading of FL (p=0.03) as 26/29 (89.7%) of BCL2 positive FL cases were of low grade (GI&GII), and 15/21 (71.4) were of high-grade (GIIIA, GIIIB), while 3/29 (10.3%) of BCL2 negative FL cases were of low grade (GI&GII), and 6/21 (28.6%) were of high-grade (GIIIA, GIIIB). This coincides with the results of translocation. Expression of BCL2 in FL showed statistically significant association with IPI score (p value=0.001) and performance (p value=0.016) with no significant association to other clinicopathologic parameters including age, gender, staging, extranodal involvement, BM or CNS involvement, anemia, LDH, B symptoms, response to therapy and relapse.

OS of BCL2 positive (mean ± SD=39.88± 13.36) was statistically favorable than OS of BCL2 negative FL (mean ± SD=5.89± 13.18, p=0.001). No statistically significant difference between the two groups regarding PFS.

DISCUSSION

FL is a heterogeneous disease including many different subgroups, as in terms of age of onset, involved organ (especially extranodal sites) and genetic abnormality. Variations of clinical course occur in FL. Some cases are very indolent, but others are not. The latter cases show histological transformation to diffuse large B-cell lymphoma (DLBCL) (high-grade transformation) and an aggressive course [20].

The IGH/BCL2 rearrangement is a relatively specific molecular marker of FL [16]. However, There is no known gold standard technique for detecting t(14;18), and a combination of southern blot (SB), conventional cytogenetics and PCR techniques is generally used [10].

Initial molecular tests were based on SB techniques which is highly sensitive, but is labor-

intensive and time-consuming, requiring the use of radioactive isotopes and high-quality DNA, which cannot be obtained from paraffin-embedded tissue specimens [9]. Similarly, performing conventional cytogenetics for the t(14;18) (q32;q21)–IGH/BCL2 in FL has several disadvantages. First, conventional chromosomal analysis requires fresh tissue. Second, successful chromosomal analysis of low-grade non-Hodgkin lymphomas often is hindered by low yields of viable metaphases. Third, karyotypes from low-grade non-Hodgkin lymphomas may be falsely negative because metaphases often are derived from normal cells rather than tumor cells. Fourth, unilateral chromosomal analysis is incapable of differentiating the t(14;18)(q32;q21)–IGH/BCL2 from the t(14;18)(q32;q21)–IgH/MALT1 associated with extranodal marginal zone B-cell lymphoma of mucosa-associated lymphoid tissue (MALT lymphoma). This can present a significant problem for the hematopathologist, because FL and MALT lymphoma may be difficult to distinguish by routine morphologic and immunophenotypic analysis [21].

The chromosomal translocation t(14;18) (q32;q21) induces BCL2 protein expression, that is identified immunohistochemically using the routine pathology diagnostic algorithms; however, a small number of cases lack BCL2 protein expression, despite carrying the t(14;18)(q32;q21) during immunohistochemistry technique [22]. In such cases, FISH and/or PCR analysis may be needed to confirm the pathologist's diagnosis.

It is clear that FISH is a more time-consuming technique than PCR. PCR is faster than other molecular methods, can be performed with poor-quality DNA, and has high sensitivity, allowing the use of archival material such as formaldehyde-fixed paraffin-embedded samples. When the results cannot be obtained by PCR due to laboratory conditions and sample quality, FISH can be used [23].

We investigated the frequency of the BCL2 breakpoints using RT-PCR and found that MBR was the most frequent breakpoint (64%). After MBR, icr was the most frequent breakpoint. Breakpoints at the icr cluster comprised 16% of our cases, significantly higher than the currently clinically used mcr breakpoint, noted to be present in only 6% of our cases. Remarkable consistency was observed when comparing our results with that obtained by Weinberg et al. (2007) [16]. In addition, 7 samples (14%) remained negative with all primers used in this study, but we could not consider these samples as true negative despite confirmation by histopathological examination, because these samples may have other minor breakpoints that were not included in this study [16]. In addition, DNA extraction from formaldehyde-fixed paraffin-embedded tissues could produce degraded DNA, which may provide another explanation for these negative cases [23].

Regarding BCL2 expression in FL in our study, 41(82%) of cases expressed BCL2, with only 9 (18%) were negative, meaning that BCL2 positivity is not pathognomonic for diagnosis of FL. These findings were in agreement with Swerdlow et al. (2008) [11] and Masir et al. (2009) [22]. Also, this was in concordance with significant correlation between BCL2 and grading of FL (P=0.001) as increasing grade of FL was associated with decreased expression of BCL2, as 11% (1/9) of grade I cases were negative, 10% (2/20) of grade II

were negative, however 28.5% (6/21) of GIII were negative. This finding goes hand in hand with that reported negative BCL2 in FL with grade III FL by lai et al. (1998) [24]., and Swerdlow et al. (2008) [11].

In this study, we reported 39/43 (90.7%) cases of t(14;18)-positive FL that were positive for BCL2 with only 4/43 (9.3%) of the t(14;18)-positive FL showing negative BCL2 expression. Similar results obtained by Skinnider et al. (1999) [25] and Masir et al. (2009) [22] who reported respectively 89% and 79% BCL2 positivity in t(14;18)-positive FL. These results coincide with the reported induction of BCL2 protein expression that occur with t(14;18), therefore most t(14;18)-positive FL were positive for BCL2. The minority of t(14;18)-positive FL that were BCL2 negative may be explained by somatic mutations of the translocated BCL2 gene, creating amino acid substitutions that prevent target epitope recognition by BCL2 antibodies during immunohistochemistry technique [13,22].

On the other hand, 2/7 cases (28.6%) of t(14;18)-negative FL, were positive for BCL2 and 5/7 cases (71.4%) were BCL2 negative. Similar results obtained by Skinnider et al. (1999) [25] who found that 25% of follicular lymphomas lacking the t(14;18) showed BCL2 immunoreactivity.. Horsman et al. (2003) [26] identified two subgroups within t(14;18)-negative FL, namely one with supernumerary chromosomes 18 and overexpression of BCL2 and another group with the frequent presence of the t(3;14)(q27;q32) involving BCL6 and no BCL2 expression. In a subset of these cases, BCL2 protein may be over-expressed by other genetic events, such as gains or amplifications of the BCL2 gene locus. In truly BCL2-negative FL, rearrangements of the BCL6 gene in 3q27 may provide an alternative molecular mechanism that can substitute for the lack of BCL2 expression. Nevertheless, the search for genetic or epigenetic hallmark alterations in t(14;18)-negative FL will have to continue [26].

Regarding correlations between translocation and histological grade, a statistically significant association of histological grade with t(14;18) and the different breakpoints was observed, with the translocation and all the breakpoints MBR, icr and mcr being more frequent in those with lower histological grade (grades I and II). This was in concordance with the previously reported studies of Lopez-Guillermo et al.(1999) and Weinberg et al. (2007) [16,27], whereas such an association was not found in a previously reported study of Buchonnet et al. (2002) [28]. Lai et al. (1998) [24] and Swerdlow et al., 2008 [11] reported negative BCL2 in grade III FL. In addition, the detected association of t(14;18) and the histological grade coincides with the current study' reported finding of the association of BCL2 expression with the histological grade, being more frequent in those with lower histological grade (grades I and II), like the t(14;18). This can be explained by the already mentioned increase BCL2 expression with t(14;18) [2].

This study revealed that there were no significant differences could be detected regarding clinical characteristics according to the breakpoint location. In contrast, Buchonnet et al. (2002) [28] reported that there was a more aggressive clinical presentation in the group of patients with a breakpoint at 5'mcr. It is worth to be mentioned that

5'mcr breakpoint was not included in this molecular assay.

One of The most effective tool for predicting outcome of patients with follicular NHL is the IPI [19]. In this study, the expression of BCL2 in FL cases showed statistically significant association with IPI score and performance status. In contrast to Lopez-Guillermo et al. (1999) [27], Logsdon et al. (1999) [29] who stated that BCL2 expression has not been associated with prognosis in patients with FL. This difference could be explained by difference in number of cases.

Complete remission in response to therapy occurred in only 18% of our cases. Also in this study, the t(14:18) and its target BCL2 values had no impact on clinical response to therapy, This cope with Schuetz et al. (2012) [30] who reported that there was not significant relation between BCL2 and patient outcome. However, Heiser et al. (2004) [31] mentioned that BCL2 expression is associated with poor response to therapy

We reported relapse in 20% of our cases. This finding was consistent with that reported by Ito et al., (2013) [32]. However, this was in controversy with Montoto et al. (2002) [33] who reported 47% relapse. In our study, 70% of relapsed cases were BCL2 positive and 80% were translocation positive. This could be explained by the fact that BCL2 is antiapoptosis protein, which makes the tumor cells live longer and become dormant resulting in failure of complete remission and increases incidence of death [31]. In this study, we found no significant correlation between PFS and translocation or BCL2 expression. This was in contrast to results of Colomo et al. (2003) [34] and de Jong et al. (2003) [35] who found that the PFS rates were significantly lower in patients whose tumors expressed BCL2. Different number of cases and follow up may explain this controversy. Therefore, further studies on larger series and longer follow up period are recommended.

In concern to OS, follow up of FL cases revealed mean OS of 33.76± 18.65 months. This finding is much lower than the total survival time reported by Szczuraszek et al. (2008) [36] which was 40 months but the relatively shorter follow up period in the current study can explain this difference. Also this difference may be related to the used therapy regimen .We detected statistically significant correlation between t(14:18) and Bcl2 expression and OS (p value=0.04, p value= 0.001 respectively). These results were in agreement with Johnson et al. (1995) [37] who found BCL2 rearrangement to be a favorable factor for survival. However, Lianos et al. (2001) [38] found that the presence of a BCL2 rearrangement has been related to a poor outcome. We also observed low disease stage among t(14;18) positive cases. However, t(14;18) negative cases presented with advanced stage (stage IIIA,IIIB)

In conclusion, a significant association of the t(14;18) with BCL2 protein expression, grading of FL, and the OS was revealed. In addition, a significant association of BCL2 protein expression with the grading of FL, OS, IPI score and performance status was detected in this study. However, further studies on larger number of patients, other minor breakpoints and longer follow up periods are recommended.

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