Original Research Article

BHRF1-1 MicroRNA of Epstein Barr Virus in Chronic B- Lymphocytic Leukemia Patients

Abstract

Background: Epstein–Barr Virus (EBV) is one of the human gamma herpes viruses that promotes subclinical and latent infections in healthy B lymphocytes. This research objects to find out the value of expression of BHRF1-1 microRNA of EBV in chronic B- lymphocytic leukemia (B-CLL) patients.

Methods: This retrospective research involved 40 subjects diagnosed with B-CLL and positive for Epstein Barr Virus Nuclear Antigen-1 IgG (EBNA-1 IgG) and 40 healthy volunteers serving as the control group. Subjects were allocated equally into two groups: group I: 40 patients with B-CLL andgroup II: 40 healthy volunteers matched for age and sex with patients serving as a control group.

Results: There was a significant increase in BHRF1-1 expression in CLL cases than control group (P value <0.001). Overall survival was significantly higher in patients with low BHRF1-1 expression (<3.63) than the patients with overexpression of BHRF1-1 in B-cellsthat was related to shorter OS BHRF1-1 expression (>3.63) (p value 0.004). Lymphocytecount (≥50 $x10^9$/L), CD38 Expression, Rai Staging (III/ IV), FISH (17p deletion vs del 13q14,trisomy 12 and del 11q23) and relative expression of BHRF1-1(high) were the significant indicators.

Conclusions: EBV-encoded miRNAs are highly found in EBV-associated tumors asBHRF1-1 MicroRNA marker of EBV.

Keywords: BHRF1-1 MicroRNA, Epstein Barr Virus, Chronic B- Lymphocytic Leukemia

Introduction:

Epstein–Barr Virus (EBV) is one of the human gamma herpes viruses that promotes subclinical and latent infections in healthy B lymphocytes. It is linked to a range of B-cell lymphomas in people with apparent cellular immunity deficiencies or without [1].

Chronic Lymphocytic Leukemia (CLL) is the major form of leukemia in humans and may manifest in destructive or inert types. Both of which are distinguished by aggregation of CD5+ Blymphocytes. Indolent CLL cases often do not need treatment for many years. Most CLL cases with aggressive form need prompt therapy. Furthermore, cases of benign CLL may evolve to the aggressive form, so it is critical to discover novel indicators for a precise early diagnosis and disease stage of CLL [2].

MiRNAs are a recent category of endogenous small noncoding RNAs that are highly related with various cancer types [3] in addition to being engaged in different cellular mechanisms, such as DNA methylation, differentiation, cellular development, and apoptosis [4, 5]. The Expression profile disclosed that miRNA can discriminate between normal B cells and malignant cells. Furthermore, miRNAs are involved in the prognosis, progression, treatment resistance, and Bcell receptor (BCR) enhancement [6, 7].

EBV miRNAs map 2 regions of the viral genome: BHRF1 and BART miRNAs. BHRF1 miRNAs are present directly upstream and downstream regions of the open reading frame of the BHRF1. BART miRNAs are present in the intronic regions of the BART genes [8]. EBV infection may affect the multiple cellular miRNAs' expression [9]. Curiously, it has been noted for the cellular miRNA, miR-155 is most strongly enhanced after lymphoblastoid B-cells EBV infection [10].

Prognostic indicators have been recognized like the un-mutated immunoglobulin heavy variable genes´ expression (UM-IgVH) and also higher concentrations of 70-kD zeta-associated protein (ZAP70) expression. Both are connected with a poor prognosis [11, 12]. Moreover,

chromosomal aberrations are involved in >80% of CLL cases and could segregate cases into various consequences. Patients with normal karyotype or have 13q deletion are low threated, while CLL cases of 11q deletion or trisomy 12 are of intermediate-risk and the high-risk patients show complex aberration or 17p deletion [13].

This research to find out the value of expression of BHRF1-1 microRNA of EBV in chronic B- lymphocytic leukemia (B-CLL) patients.

Meterials and Methods:

This retrospective research involved 40 subjects diagnosed with B-CLL and positive for Epstein Barr Virus Nuclear Antigen-1 IgG (EBNA-1 IgG)and 40 healthy volunteers serving as control group.

Exclusion criteria were any other lymphoproliferative disorder.

Subjects were allocated equally into two groups: group I: 40 subjects with B-CLL and group II: 40 apparently healthy volunteers matched for age and sex with patients serving as control group.

All subjects underwent full history taking and complete clinical investigation, abdominal ultrasonography, laboratory investigations (CBC, flow cytometric analysis, immunophenotyping) and specific laboratory tests (ELISA and quantitative-real time PCR).

Sample collection:

Data extracted were recorded after the following:

Peripheral blood (PB) specimens were collected from each case into sterile vacutainers and were divided as follows:

Approximately 4mL was obtained in EDTA-containing tubes and processed directly after obtained for CBC (Abbott Cell Dyn Ruby Hematology Analyzer) and also for multiparameter flow cytometry (MFC). 2mL was collected in EDTA-containing tubes and sent to the Cell Separation Service for cell sorting. 3 mL was collected in plain tube then centrifuged; sera were stored at -20°C till the time of EBNA-1 IgG measurement.

Flow cytometry immunophenotypic studies

FC is the method of passing cells individually in a fluid stream via a light beam. This fluid stream is identified by laminar flow with high flow rate and is called isotonic sheath fluid. Flow cytometers measure the quantity of light that is emitted by fluorochromes associated with individual cells or particles. Flow cytometers have three to four well defined lasers. Antibodies are conjugated with fluorochromes. These fluorochromes are pigments that absorb light from the laser that will be emitted with longer wavelengths.

Flow cytometer consists of three main compartments: fluid transport molecules in a flow to the laser beam, an optic system with lasers to illuminate molecules in the specimen stream, and optical filters for direction the generated light signals to a suitable detector.

Fluorescence-activated Cell Sorting (FACS) purification of B-cell Populations for all individuals studied

Regarding to the CLL patients, PB B-CLL cells were sorted by a FACSAria III flow cytometer (BD). B-CLL cells were kept based on their distinctive light scatter characteristics and twofold positive for CD19 and CD5. In samples (n=40) with higher than one defective B-cell population, differentiation was dependent on their discrete styles of expression for at least one of the examined B-cell indicators. Clonality of every FACS-purified B- cell population (final purity of 98% ± 0.8%) was verified using the FACSDiva (BD) software, and about 1 ml of these sorted B-CLL cells, besides sorted B-cells the from healthy volunteers were split into aliquots and sent to the national DNA bank (Salamanca university) for storage(at −80 °C) and further studies (e.g., cytogenetic and molecular studies), which were described below [14, 15].

Measurement of serum level of EBNA-1 IgG

The Serum level of EBNA-1 IgG was determined by commercial ELISA Kit (company: Fitzgerald; catalogue number: 20-EG46) for the quantitative detection of EBNA-1 IgG.

Flow cytometric detection of Zeta-chain associated protein 70 (ZAP-70)

The Sorted aliquoted B-CLL cells (CD19+ CD5+) were obtained from the national DNA bank (Salamanca university) for flow cytometric detection of (ZAP-70). The cells were thawed, then fixed and permeabilized by the Fix and Perm kit [16]. 5 µg of antiZAP-70 monoclonal antibody was added to cells, and the mixture was incubated for 20 minutes at room temperature; the cells were washed twice in PBS, incubated for 20 minutes with goat antimouse immunoglobulin-FITC (DAKO), washed, then incubated with normal mouse serum (DAKO) for 5 minutes. After that, the specimens were obtained on a FACSCanto II FC (BD) utilizing the FACSDiva software (BD). A case was deemed ZAP 70 positive if ≥ 20% of the B-CLL cells expressed ZAP 70 [12].

Interphase Fluorescence in Situ Hybridization (iFISH) Studies

The sorted CD19+/CD5+ leukemic B-cells were obtained from the national DNA bank (Salamanca University) for FISH studies. FISH is an efficient method for identifying chromosomal abnormalities. FISH permits patient categorization into good (deletion 13q14), intermediate (trisomy 12), and poor (deletion of ATM and deletion of TP53) prognostic groups for chronic lymphocytic leukemia/small lymphocytic lymphoma, in addition to monitoring disease progression. Components subblied by Vysis CLL FISH Probe Kit.

The samples were analyzed by fluorescent microscopy using red, green and DAPI filters. The cut off levels for trisomy 12 (>2%), del (11q22.3) (>4%), del (11q23.3) (>2%), del (17p13.1) (>7%), and del(13q14) (>4%) were formed based on iFISH patterns **(Wiktor et al., 2006; Dohner et al.,2000).**

Molecular Studies (quantitative-real time PCR)

The Sorted B-cells were obtained from the national DNA bank (Salamanca University), then RNA was extracted for quantitative-real time PCR studies for the expression of BHRF1-1 MicroRNA of EBV in B-lymphocytes. Real-time qPCR analysis was done by gene-specific Taqman assay, with a CFX384 real-time PCR detection system, both the forward and reverse primers.

In summary, in a total volume of 10 mL, 20 ng/mL cDNA product was added to a master mix, containing 10 pmol/mL of miR-BHRF1-1 and normalizer 5SrRNA (reference gene) primers.

The cycle settings for EBV-miR-BHRF1-1 and 5Sr RNA were as follows: one round for 3 minutes at 95°C, forty rounds for 12 seconds at 95°C, and forty seconds at 62°C; melting curves were monitored concurrently. Relative expression concentration of a gene was determined by the formula $2^{-\Delta Ct}$ using the Applied Biosystems ABI Step One Plus Real-time PCR system.

Sample Size Calculation:

Utilizing open Epi Program with confidence level 95% and power 80% and doing a pilot studyover 10 cases and 10 control and we found that the mean of BHRF1-1 expression in cases was 2.73+/-2 compared to 1.08+/-0.69 and we found the sample size was 40.

Statistical analysis

Statistical analysis was performed by SPSS v25 (IBM Inc., Chicago, IL, USA). Quantitative data were described as mean and standard deviation (SD) and were analyzed by paired Student's t- test for the same group. Qualitative data were described as frequency and percentage (%) and were analyzed by Chi-square test. Nonparametric data were analyzed by Mann Whitney test. P value < 0.05 was deemed significant.

Results:

There was an insignificant variance in sex and age. There was a significant decrease in Hb level and PLT count and a significant increase in TLC, peripheral lymphocytes, ALC and BHRF1-1 expression in CLL patients compared to control group (P value <0.001).

Table 1: Demographic data, laboratory data and BHRF1-1 expression of the studied patients

		CLL Patients (n = 40)	Control (n = 40)	p
Sex	**Male**	26 (65.0%)	27 (67.5%)	0.813
	Female	14 (35.0%)	13 (32.5%)	
Age (years)	**Mean ± SD.**	63.78 ± 9.16	61.15 ± 11.30	0.257
Hb (g/dl)	**Mean ± SD.**	10.48 ± 1.94	14.08 ± 1.48	<0.001*
PLT (x10^9/L)	**Median (IQR)**	113.0 (83.50 – 190.0)	279.0 (215.0 – 330.0)	<0.001*
TLC (x10^9/L)	**Median (IQR)**	74.0 (36.90 – 225.0)	7.90 (6.38 – 9.38)	<0.001*
Lymphocytes (%)	**Mean ± SD.**	68.25 ± 16.45	32.98 ± 6.94	<0.001*
LC (x10^9/L)	**Median (IQR)**	53.37 (29.14 – 146.40)	2.24 (1.85 – 3.19)	<0.001*
BHRF1-1 expression	**Median (IQR)**	3.63 (2.12 – 4.44)	0.31 (0.13 – 0.81)	<0.001*

Data are presented as mean ± SD, Median (IQR) or frequency (%). Hb: hemoglobin, PLT: platelet count, TLC: Total Leukocyte Count, ALC: Absolute Lymphocyte Count, *: Statistically significant at $p \leq 0.05$

Table 2 shows distribution of the studied cases according to different parameters in CLL group.

Table 2: Distribution of the studied cases according to different parameters in CLL group (n=40)

Parameter		No.	%
Rai Staging	**0**	5	12.5
	I	7	17.5
	II	16	40.0
	III	6	15.0
	IV	6	15.0
Lymphadenopathy		26	65.0
Organomegaly (splenomegaly and/or Hepatomegaly)		18	45.0
Hb (<11) g/dl		16	40.0
PLT (<100) (x10^9/L)		14	35.0
Lymphocytosis		40	100.0
ZAP70 Expression		15	37.5
CD38 Expression		14	35.0
Del 13q14		26	65.0
Del 17p13		8	20.0
Trisomy 12		15	37.5
Del 11q23		10	25.0

Data are presented as frequency (%).

Figure 1 shows the Kaplan-Meier survival curve with a significant higher overall survival in cases with low BHRF1-1 expression (<3.63) than those with overexpression of BHRF1-1 in

B-cells that was related with shorter OS BHRF1-1 expression (>3.63), (p value0.004).

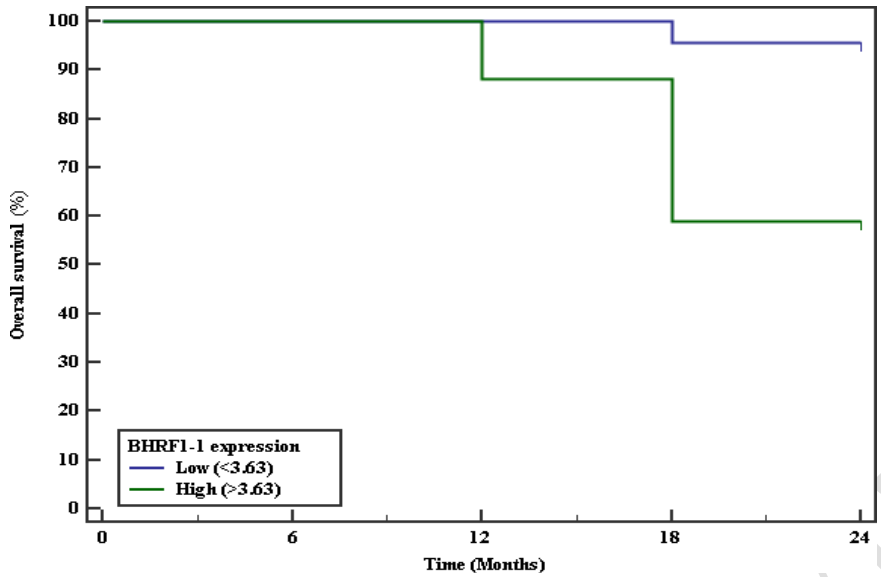


Figure 1: Kaplan-Meier survival curve for overall survival with BHRF1-1 expression

There was a significant relation between CLL patients age (more than 65 years), lymphocytes ≥50 ($x10^9$/L), stage III+IV of Rai and staging positive cases of CD38 Expression and between high BHRF1-1 expression (>3.63) (p value= 0.005, 0.030, 0.043 and <0.001 respectively). In according to FISH studies there was significant relation between and Positive cases of del 17p13 and high BHRF1-1 expression (>3.63), (p value 0.006).

Table 3: Relation between BHRF1-1 expression and clinical parameters in patients with CLL (n= 40)

		BHRF1-1 expression		p
		Low (<3.63) (n= 23)	High (>3.63) (n= 17)	
Age	**≤65**	17 (73.9%)	5 (29.4%)	0.005*
	>65	6 (26.1%)	12 (70.6%)	
Sex	**Male**	13 (56.5%)	13 (76.5%)	0.191
	Female	10 (43.5%)	4 (23.5%)	
Lymphocytes($x10^9$/L)	**<50**	3 (13.0%)	8 (47.1%)	FEp= 0.030*
	≥50	20 (87.0%)	9 (52.9%)	
Lymphadenopathy	**No**	6 (26.1%)	8 (47.1%)	0.169
	Yes	17 (73.9%)	9 (52.9%%)	
Organomegaly (splenomegaly and/or Hepatomegaly)	**No**	12 (52.2%)	10 (58.8%)	0.676
	Yes	11 (47.8%)	7 (41.2%)	
Rai staging	**Stage 0+I+II**	19 (82.6%)	9 (52.9%)	MCp 0.043*
	Stage III+IV	4 (17.4%)	8 (47.1%)	
ZAP70 Expression	**Negative**	15 (65.2%)	10 (58.8%)	0.680
	Positive	8 (34.8%)	7 (41.2%)	
CD38 Expression	**Negative**	22 (95.7%)	4 (23.5%)	<0.001*
	Positive	1 (4.3%)	13 (76.5%)	
Del 13q14	**Negative**	7 (30.4%)	7 (41.2%)	0.481
	Positive	16 (69.6%)	10 (58.8%)	
Del 17p13	**Negative**	22 (95.7%)	10 (58.8%)	FEp= 0.006*
	Positive	1 (4.3%)	7 (41.2%)	

Trisomy 12	**Negative**	15 (65.2%)	10 (58.8%)	0.680
	Positive	8 (34.8%)	7 (41.2%)	
Del 11q23	**Negative**	16 (69.6%)	14 (82.4%)	$^{FE}p=$ 0.471
	Positive	7 (30.4%)	3 (17.6%)	

Data are presented as frequency (%), *: Statistically significant at $p \leq 0.05$

Lymphocyte count (≥50 $x10^9$/L), CD38 Expression, Rai Staging (III/ IV), FISH (17p deletion vs others) * and relative expression of BHRF1-1(high) were the significant indicators.

Table 4: Univariate and multivariate COX regression analysis for the parameters

	Univariate		**#Multivariate**	
	p	**HR (95%C.I)**	**p**	**HR (95%C.I)**
Age (>65)	**0.089**	4.088(0.809 – 19.865)		
Sex (male)	**0.194**	4.004(0.493 – 32.550)		
ALC ($x10^9$/L) (>53.37)	**0.149**	3.248(0.655 – 16.099)		
Lymphocyte count (≥50)	**0.004***	0.046(0.006 – 0.378)	0.197	0.184(0.014 – 2.405)
CD38 Expression	**0.012***	14.617(1.798 – 118.864)	0.505	6.264(0.028 – 1385.22)
ZAP70 Expression	**0.382**	1.856(0.464 – 7.424)		
Rai Staging (III/ IV)	**0.011***	8.038(1.621 – 39.862)	0.565	1.822(0.236 – 14.056)
BHRF1-1 expression (high)	**0.028***	10.414(1.281 – 84.670)	0.970	1.110(0.005 – 258.001)
FISH (17p deletion vs others)*	**0.020***	5.171(1.293 – 20.688)	0.103	3.399(0.780 – 14.803)

: Statistically significant at $p \leq 0.05$, (Others) = del 13q14, trisomy 12 and del 11q23

Figure 2: Flow cytometric phenotype of chronic lymphocytic leukemia (CLL). The neoplastic B cells (red) are positive for CD19, CD43, CD23, CD200, dim intensity for CD20 and CD79b

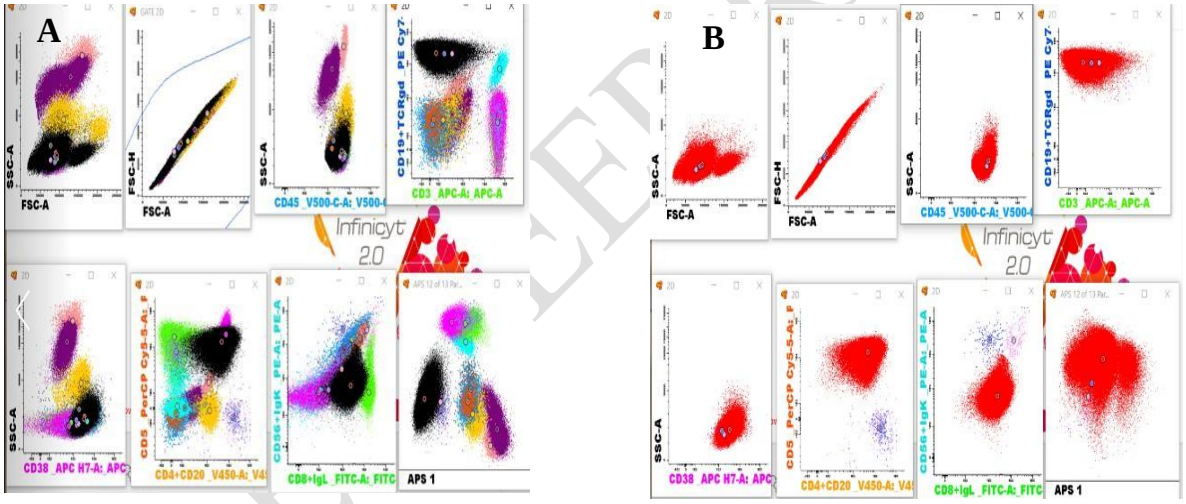
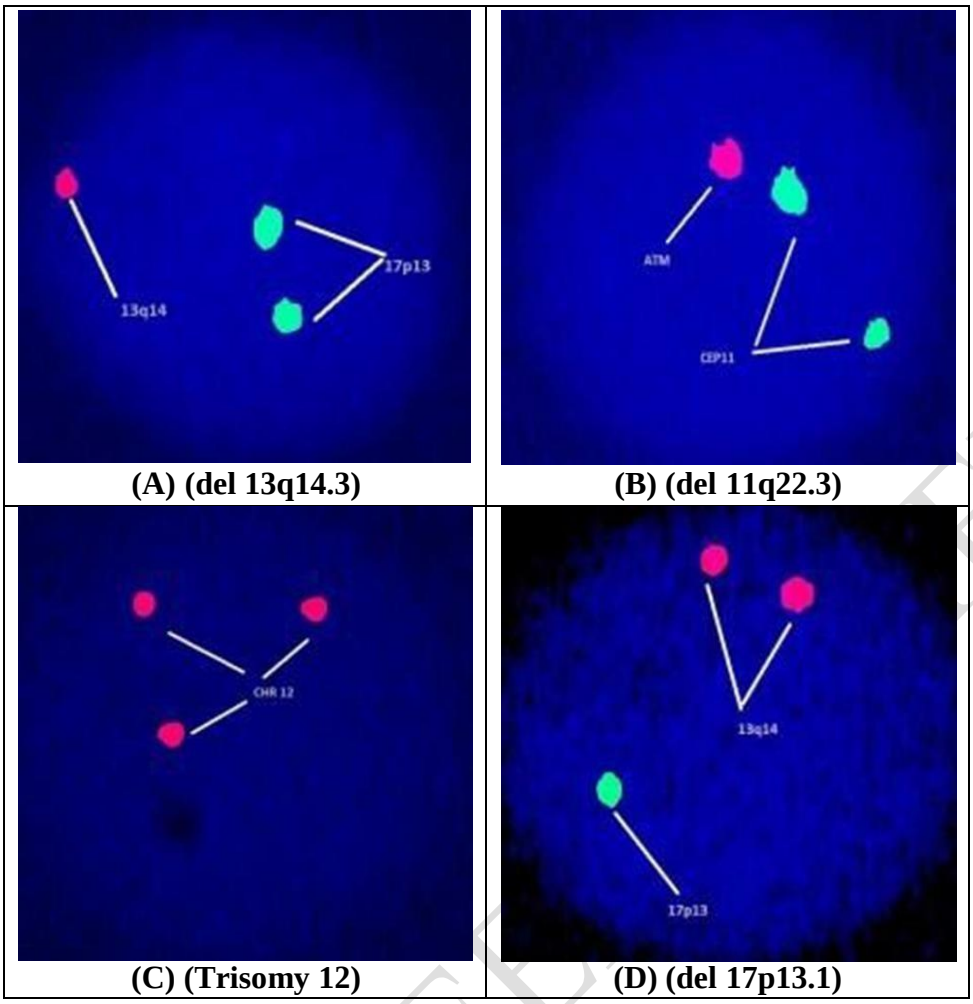


Figure 3: Flow cytometric phenotype of chronic lymphocytic leukemia (CLL). The neoplastic B cells (black A, red B) are positive for CD19, CD5, CD38, and show restricted lambda immunoglobulin light chain expression



(A) (del 13q14.3) **(B) (del 11q22.3)**

(C) (Trisomy 12) **(D) (del 17p13.1)**

Figure 4: Detection of cytogenetic abnormalities in CLL using fluoresence in situ hybridiztion technique

Discussion

EBV is a pervasive carcinogenic human herpesvirus involved in lymphomas like Burkitt's lymphoma, while newer research has linked EBV infection with the advancement of B-CLL [17, 18].

In the present research, the Hb levels of the majority of CLL cases were significantly lower than those of the control group. This finding was in line with those of Littlewood and Mandelli, [19], Sagatys and Zhang [20] who reported the decrease in Hb in CLL cases due to either hemolysis,bone marrow infiltration, and/or splenic sequestration of red blood cells.

Also, there was significant decline in PLT count in CLL cases than control ones. This was consistent with Parker et al. [21] who reported that decrease platelets count in CLL patients due to marrow replacement and hypersplenism.

Our study revealed that the TLC demonstrated significant elevation in cases with CLL as in comparison to control group. In consistent with these findings, Palumbo et al. [22] reealed leukocytosis in CLL cases. Moreover, our study revealed that the mean value of ALC in peripheral blood found significant increase in CLL cases in contrast to the controlgroup which was compatible with Perry et al. [23] who reported that CLL patients showed higher absolute lymphocytosis compared to control group due to accumulation of B-cell.

According to Rai staging system as a part of this study, CLL patients were categorized into five classes (0, I, II, III, IV) and most of them were included in low and intermediate risk groups (I, II) with lymphocytosis together with lymphadenopathy and /or organomegaly (splenomegaly and/or Hepatomegaly), this was consistent with Wu et al. [24] who reported most CLL cases were low risk. Also, Sagatys and Zhang [20] demonstrated that According to the modified Rai staging method, 80% of newly diagnosed CLL patients had a low risk.

In this research, FACS sorting (purification) was done to assess the quality of the B-CLL and for subsequent qPCR analysis, which offers a potent combination of techniques that permits gene expression profiling of such malignant cells (malignant B-cells; B-CLL). We discovered that the levels of EBV miRNA BHRF1-1 expression in the B- cells of CLL cases were substantially greater than in healthy ones. The expression of BHRF1-1 in CLL cases ranged from 0.31 – 9.0 with a median (IQR) of 3.63(2.12 – 4.44) and in control group from 0.02 – 1.30 with a median (IQR) of 0.31 (0.13 – 0.81), that in line with similar findings demonstrated by Visco et al. [25] and Xu et al. [26] who foundCLL patients had greater EBV-BHRF1-1 levels than the healthy control group. This finding is consistent with earlier research and is owing to its essential involvement in B-cell differentiation and the prevention of apoptotic processes.

We found a significant higher overall survival in cases with low BHRF1-1 expression than the cases with overexpression of BHRF1-1 in B-cells was linked with shorter OS. These findings are comparable to those of Liang JH et al. [27], who found that elevated concentrations of EBV-BHRF1-1 were related with poor survival in CLL cases. These findings designate the possible significance of this miRNA as a biomarker.

We studied the Relation between BHRF1-1 expression and clinical parameters in patients with CLL and we found the following:

There was a significant relation between CLL patients¸ age (more than 65 years), Lymphocytes ≥50 ($x10^9$/L) and high BHRF1-1 expression (>3.63), (p value= 0.005 and 0.030 respectively).

We also found a significant relation between Stage III+IV of Rai staging and high BHRF1-1 expression (>3.63), in B-CLL patients (p value= 0.043).

In our study we found that statistically significant relation (p value <0.05) between the Lymphocyte count of ≥50 $x10^9$/L, positive CD38 Expression, Rai Staging (III/ IV), FISH (17p deletion vs others) and the high levels of BHRF1-1 expression (>3.63).

In according to our study to FISH findings, we found that there was significant relation between positive cases of del 17p13 and high BHRF1-1 expression (>3.63), (p value 0.006). Xu et al., [26] reported higher concentrations of EBV-BHRF1-1 were related with decreased concentrations of p53, indicating that EBV-BHRF1-1 may play a role in the events that contribute to CLL development.

We did a univariate COX regression analysis, and we found that the cases with high BHRF1-1 expression levels had an increased risk of mortality than those with low relative expression. Similar result was observed for Rai Staging (III/ IV).

As well as the patients with Lymphocyte count (≥50 x10^9/L) had an increased risk of mortality. Similar result was observed for CD38 Expression and FISH (17p deletion vs others).

Conclusions:

EBV-encoded miRNAs are highly presented in EBV-associated tumors as the BHRF1-1 MicroRNA marker of EBV. Management of the activity of EBV-encoded miRNAs in EBV-positive tumours may be a viable technique for repairing host anti-tumor immunity, providing a unique treatment approach for EBV- associated tumors.

Ethical Approval

The research was applied after approval from the Ethical Committee of Tanta university (32063/01/18), in collaboration with Salamanca university (USAL),Spain.

Disclaimer

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The Thesis document is available in this link: https://www.researchgate.net/profile/Ahmed-Elgohary-4

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