OncoTargets and Therapy

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ORIGINAL RESEARCH

Overexpression of *BCL2*, *BCL6*, *VEGFR1* and *TWIST1* in Circulating Tumor Cells Derived from Patients with DLBCL Decreases Event-Free Survival

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Purpose: Diffuse large B-cell lymphoma (DLBCL) is a heterogeneous malignant lymphoid neoplasm and is the most common subtype of non-Hodgkin lymphoma in adults. More than half of patients with DLBCL can achieve remission with standard R-CHOP regimes; however, approximately 30–40% of patients are still failing this standard therapy, which remains as an important cause of progression and mortality of this disease. It is necessary to have diagnostic and monitoring tools that allow us to improve the accuracy of prognosis in these patients. Circulating tumor cells (CTCs) identification through molecular biomarkers is one of the novel strategies that have been used in other types of cancer, and we aim to use this tool to analyze the potential role in DLBCL.

Patients and Methods: We analyzed 138 blood samples of patients with DLBCL, of which CTCs were isolated by density gradient for subsequent detection and quantitation of molecular biomarkers using RT-qPCR with TaqMan probes. Survival analysis was performed using Kaplan–Meier curves.

Results: We found overexpression of *ABCB1, αSMA, BCL2, BCL6* and *VEGFR1* genes, as well as the presence of *CK19, EpCAM, KI67, MAGE-A4, SNAIL* and *TWIST1* genes. *CK19* and *EpCAM* expression were associated with a minor OS (85.7% vs 98.1%, p = 0.002). The overexpression of *BCL2, BCL6, VEGFR1* and *TWIST1* was related to a minor EFS (p = 0.001).

Conclusion: This study showed that in liquid biopsies analyzed, the presence of CTCs can be confirmed through molecular biomarkers, and it has an impact on OS and EFs, making this detection useful in the follow-up and prognosis of patients with DLBCL.

Keywords: biomarkers, CTCs, lymphoma, liquid biopsies

Introduction

Diffuse large B-cell lymphoma (DLBCL) is the most frequent type of lymphoma in Mexico (approximately between 30% and 50% of the total new cases).[1,2] DLBCL represents a heterogeneous group of tumors with high variability in genetic abnormalities, clinical characteristics, response to treatment, and prognosis.[3] Despite the advances in immunotherapy and the incorporation of new cytotoxic agents, an unfavorable prognosis still exists in a particular group of patients. This is partly due to the presence of metastatic cells that can infiltrate, survive and colonize different organs.[4,5]

Circulating tumor cells (CTCs) are released into the blood or lymphatic system from the primary tumor, which leads to the spreading of the disease to other organs and tissues. By adhering to the walls of capillaries and escaping from the blood vessel (extravasation), they can colonize organs different from the primary tumor, generating metastasis.[6,7] In various studies, it has been determined that tumor cells emerge from the primary tumor since the initial stages of

Received: 18 August 2022
Accepted: 8 December 2022
Published: 30 December 2022

malignant progression, but even in patients with advanced metastatic cancer, CTCs represent only 0.0001% of all nucleated cells, finding up to 5 tumoral cells by mL.[8,9]

Currently, the detection of CTCs in peripheral blood has gained a great relevance; therefore, very sensitive technologies that allow precise CTCs detection have been developed in last years. One of the most widely used techniques for this detection is the RT-qPCR, in which biomarkers or specific tumor genes that are not originally present in the blood of healthy individuals are detected. Different kinds of biomarkers have been used for the identification of tumors, and these in turn have been applied for the characterization of the CTCs.[8,10]

The presence of CTCs in various types of cancer has been demonstrated; numerous studies show the association of the presence of CTCs with an unfavorable prognosis in patients with melanoma and sarcoma. However, the study of CTCs and their role in the generation of metastasis have been more frequently focused in carcinomas.[10,11] Dissemination in DLBCL occurs when cancer cells originating in the lymph node migrate through the lymphatic and circulatory system to distal organs or sites far from the primary tumor.

Clinically, the DLBCL is considered an aggressive histological variant of lymphoma, presenting accelerated tumor growth at nodal and extranodal sites.

Sixty percent of patients with DLBCL are in stages III (nodal regions on both sides of the diaphragm) or IV (disseminated infiltration of one or more foreign organs involving or not lymph nodes) at diagnosis, which is an indicator of dissemination.[11,12] The detection of biomarkers involved in cancer hallmarks, combined with EpCAM and cytokeratins[13] (used in the CellSearch® system approved by FDA for the detection of CTCs)[14] is of utmost importance for the characterization of CTCs, which provides additional prognostic information for the best handling of patients with malignant neoplasms.[15] For this reason, it is essential to have a molecular-grade panel of biomarkers in liquid biopsies that allow us to gather more information about their clinical impact on DLBCL.

Materials and Methods

Study Population

A unicentric, observational, prospective study, without intervention, which consisted of taking liquid biopsies (peripheral blood) and clinical data of patients with DLBCL treated with R-CHOP of the Hospital General de México "Dr. Eduardo Liceaga" was carried out. The inclusion criteria were (1) male or female adults, (2) DLBCL diagnosis (3) treated with R-CHOP, (4) informed consent signature. A total of 138 patients with diagnosis of DLBCL confirmed by hematological studies were recruited from January 2019 to December 2021. Immunohistochemistry assays (Hans algorithm) were performed to know the cell of origin (Germinal Center B-cell, GCB and non-Germinal Center B-cell, non-GCB). Peripheral blood (PB) samples were obtained before the start of R-CHOP treatment. The clinical information was carried out prospectively. The response to the treatment was evaluated by PET-CT.

The informed written consents were collected from all enrolled patients, and the entire study was performed based on the Declaration of Helsinki.

CTC Enrichment

From each patient, 8 mL of anticoagulated blood with EDTA was obtained by venipuncture (Vacutainer tubes, BD Diagnostics Franklin Lakes, New Jersey), before the start of treatment. Blood samples were placed in a 1:2 volume (Lymphoprep: blood) and were centrifuged according to the manufacturer's protocol (Axis-Shield, Oslo, Norway). Due to their same density values (<1.077 g/mL), the fraction containing mononuclear cells and CTCs was obtained pipetting directly the upper lymphoprep layer and aliquoted in pellets of 3×10^6 cells, according to the counting performed in a Corning Cell Counter (Corning Inc., Corning, NY, USA), and homogenized in TRIzol (Invitrogen, Life Technologies Carlsbad, CA) for nucleic acid extraction.

RNA Extraction and cDNA Synthesis

The RNA was isolated by TRIzol (Invitrogen, Life Technologies Carlsbad, CA), according to the manufacturer's protocol. The concentration and purity of the RNA was determined by measuring the absorbance of the samples at

260 and 280 nm performed in a Nanodrop spectrophotometer (ThermoScientific, Wilmington, DE, USA). The integrity was corroborated by running a 1% agarose-gel electrophoresis, observing the 18s and 28s ribosomal RNA (rRNA) bands. Corroborated RNAs were stored at −80°C until use. Briefly, 2500 ng of RNA was reverse-transcribed into cDNA, using Oligo dT and the reverse transcriptase MML-V, according to the manufacturer's protocol (PROMEGA, Madison WI, USA).

Biomarkers Detection

To determine the relative expression levels of biomarkers, RT-qPCR were performed by triplicated on a Step One ™ Applied Biosystems equipment, using 250 ng of cDNA, TaqMan ™ Gene Expression Master Mix, and specific hydrolysis probes for each biomarker: *ABCB1* (Hs04992772_s1), *αSMA* (Hs00559403_m1), *BCL2* (Hs04986394_s1), *BCL6* (Hs00153368_m1), *CK19* (Hs00761767_s1), *EpCAM* (Hs00901885_m1), *KI67* (Hs04260396_g1), *MAGE-A4* (Hs00751150_s1), *SNAIL* (Hs00195591_m1), *TWIST1* (Hs04989912_s1) and *VEGFR1* (Hs01052961_m1). The amplification protocol used was 95 °C denaturation for 10 minutes and amplification and quantification for 40 cycles (95 °C for 15 seconds, 60 °C for 60 seconds). Expression levels were obtained with the 2-ΔΔCt method, using *GUSB* (Hs00939627_m1) as endogenous gene and the K562 hematological cell line (CCL-243™, ATCC) as the reference sample.

Statistical Analysis

The categorical variables were expressed through absolute proportions and values. The quantitative variables in means and standard deviations or medians and interquartile ranges, as corresponded to the normality of the data, were analyzed with the Anderson–Darling test. The Kaplan–Meier method was used for survival analysis, total mortality, EFS, and an Odds Ratio risk analysis. The statistical analysis was performed using the SPSS software version 25–0 (IBM, Armonk, NY, USA). A value of $p < 0.05$ was considered as a significant difference.

Ethical Considerations

This trial is a minimum risk investigation. For its realization, it was approved by the Ethics and Research Committees of the Hospital General de México "Dr. Eduardo Liceaga" with registration numbers DI/19/103/03/006 and DI/16/103/03/035.

Results

Characteristics of the Cohort Study

A total of 138 patients with DLBCL were included, all patients were treated with the R-CHOP first-line scheme (Rituximab 375 mg/kg, cyclophosphamide 750 mg/kg, doxorubicin 50 mg/kg, vincristine 1.4 mg/kg and prednisone 1 mg/kg, for 6 cycles, one every 21 days), of which 61 cases (44.2%) corresponded to type non-GCB and 77 (55.8%) to GCB according to the immunopathological classification. The mean age was 54 years (20–87) with predominance of the female gender (77 patients, 55.8%). Among the relevant clinical parameters, the 0–2 IPI Score was presented in 92 cases (66.7%); and as for the clinical stage, III and IV (advanced) with 88 cases (63.8%) predominated; the 0–1 ECOG were 111 (80.4%). The extra-nodal sites were detected in 60 patients (43.5%); and finally, LDH levels greater than 271 U/L were present in 52 patients (37.7%), Table 1.

Samples of 138 healthy individuals (mean age 36 years old, range between 18 and 62, 39% females and 61% males, negative viral serology, normal blood count, negative chronic diseases) were used as controls for normal expression of the biomarker panel. For *αSMA, ABCB1, BCL2, BCL6* and *VEGFR1* genes, the normal expression value was obtained according to the sum of the 95% confidence interval (CI 95) for the mean and standard deviation, thus establishing the cut-off point from which overexpression in patients' samples was considered. To compare the control group against patients, the Anderson–Darling test and *T*-test were performed to evaluate significant differences between the means of expression. The results showed overexpression of the genes *αSMA* ($p = 0.002$), *ABCB1* ($p = 0.001$), *BCL2* ($p = 0.004$),

Table 1 Clinicopathological Characteristics of the Population Analyzed (N = 138)

Clinical Features	Cases (%)	
Age	Mean (Range)	54 (20–87)
	< 60	87 (63)
	≥60	51 (37)
Gender	Male	61 (44.2)
	Female	77 (55.8)
Leucocytes	Mean (Range)	6.09 (0.4–15.90)
	Low	37 (26.8)
	Normal	84 (60.9)
	High	17 (12.3)
Hb	Mean (Range)	11.84 (6–16.8)
	<14	109 (79)
	Normal	29 (21)
Platelets	Mean (Range)	309 (20–1176)
	<150	25 (18.1)
	Normal	88 (63.8)
	≥ 450	25 (18.1)
Lactate dehydrogenase	Mean (Range)	391 (98–3196)
	<271	86 (62.3)
	≥271	52 (37.7)
Cell of origin	GCB	77 (55.8)
	Non-GCB	61 (44.2)
ECOG %	0–1	111 (80.4)
	2–4	27 (19.6)
Extranodal sites	No	78 (56.5)
	Yes	60 (43.5)
Clinical stage	I–II	50 (36.2)
	III–IV	88 (63.8)
IPI Score	0–2	92 (66.7)
	3–5	46 (33.3)
R-IPI Score	Very Good	23 (16.7)
	Good	91 (65.9)
	Poor	24 (17.4)

Abbreviations: ECOG, Eastern Cooperative Oncology Group Performance Status; Hb, Hemoglobin; GCB, Germinal Center B-cell; non-GCB, non-Germinal Center B-cell; IPI, International Prognostic Index; R-IPI, Revised International Prognostic Index.

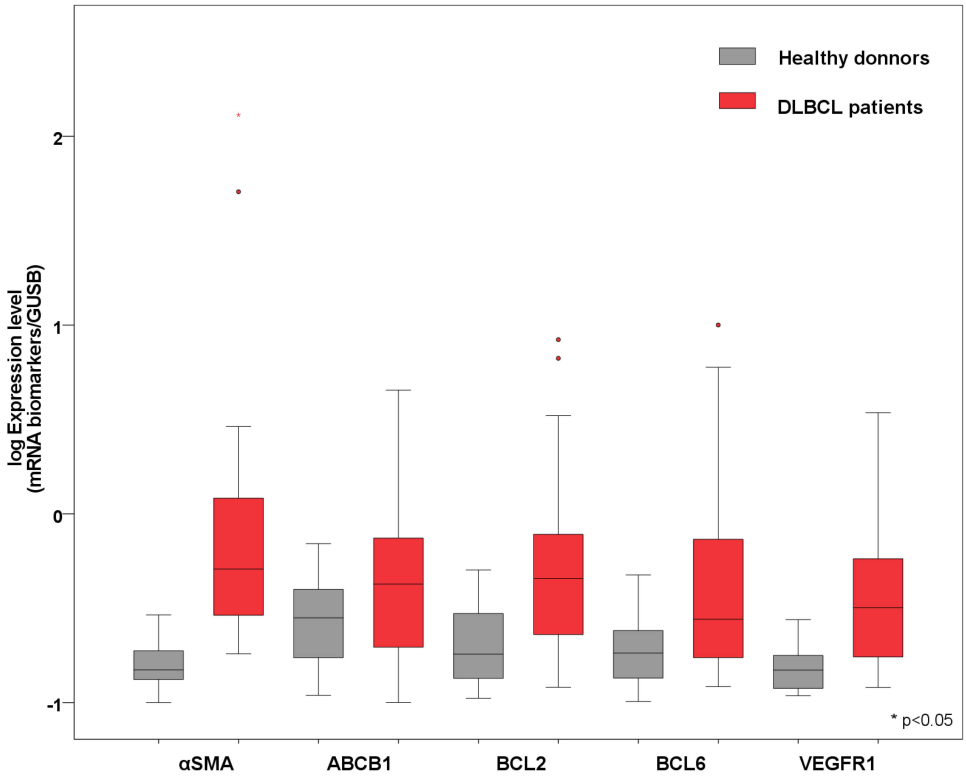

Figure 1 Differential expression of mRNA biomarkers in healthy donors (N = 138) and DLBCL patients (N = 138).

BCL6 (p = 0.016) and *VEGFR1* (p = 0.003) in the CTCs of patients with DLBCL compared to the samples of healthy individuals (Figure 1).

In the case of the *CK19, Ki67, MAGE-A4, NY-ESO1, SNAIL* and *TWIST1* genes, they had no expression in the samples of healthy individuals since its expression is specific in tumors, which some were previously analyzed for leukemia and lymphoma by our workgroup.[16,17]

Biomarkers Frequency in CTCs

When analyzing biomarkers in liquid biopsies derived from patients with DLBCL, *BCL2* and *ABCB1* genes were the most frequent with 21.7% and 28.3%, respectively. The genes that were between 10 and 20% were *EpCAM* (18.8%), *BCL6* (18.1%), *VEGFR1* (18.1%), *TWIST1* (17.4%), *αSMA* (12.3%), *CK19* (12.3%) and *Ki67* (11.6%). *MAGE-A4* and *SNAIL* genes were below 10% expression, Table 2. The overexpression percentage of *αSMA, ABCB1, BCL2, BCL6,*

Table 2 Molecular Biomarkers in CTC DLBCL Patients (N = 138)

Biomarker Status		Cases (N)	%
αSMA	Normal	121	87.7
	Overexpression	17	12.3
ABCB1	Normal	99	71.7
	Overexpression	39	28.3
BCL2	Normal	108	78.3
	Overexpression	30	21.7

(*Continued*)

Table 2 (Continued).

Biomarker Status		Cases (N)	%
BCL6	Normal	113	81.9
	Overexpression	25	18.1
VEGFR1	Normal	113	81.9
	Overexpression	25	18.1
CK19	Negative	121	87.7
	Present	17	12.3
EpCAM	Negative	112	81.2
	Present	26	18.8
Ki67	Negative	122	88.4
	Present	16	11.6
MAGE-A4	Negative	125	90.6
	Present	13	9.4
SNAIL	Negative	136	98.6
	Present	2	1.4
TWIST1	Negative	114	82.6
	Present	24	17.4

VEGFR1 and presence of *CK19, EpCAM, KI67, MAGE-A4, SNAIL* was considered as oncogenic events related to the presence of CTCs. The correlation analysis between clinicopathological variables and overexpression/presence of genes of the biomarker panel was performed without finding results of clinical relevance. The real impact of our study resides in overall and event-free survival, as described below.

Presence of CTCs in Patients and Overall/Event-Free Survival

In an average follow-up time of 485 days (9–1237) the overall survival was 94.9% (OS, the percentage of patients that remained alive from DLBCL diagnosis to death or surveillance length) (Figure 2A) and event-free survival (EFS, the percentage of patients that remains free of disease complications, death, relapse, refractory through the time of study duration) was 53.6% (Figure 2B). When analyzing the presence of CTCs and the OS, we found that patients with the presence of *EpCAM* or *CK19* biomarkers presented a worse survival of 85.7% compared to those who did not present them (98.1%, p = 0.002) (Figure 2C). As for the EFS, in those patients who did not have biomarker expression, the survival average was 1134 days (CI 1039–1229), against those who did present expression of biomarkers, whose average survival was 891 days (CI 789–999), Log Rank p = 0.018 (91.7% for patients with normal/negative gene expression vs 70.7% in patients with 1 or more biomarkers overexpressed/present) (Figure 2D).

The genes that showed an impact on EFS were *BCL2, TWIST1* and *VEGFR1*. In the case of *BCL2*, patients who had a normal expression presented an average of 751 days, CI 95% (656–846), vs patients with overexpression with an average of 558 days, CI 95% (407–704) Log Rank p = 0.030 (Figure 3A). Patients with *TWIST1* presence had a worse survival (25%) than those who did not present it (59.6%) Log Rank p = 0.007 (Figure 3B). As for the expression of the *VEGFR1* gene, patients who presented overexpression had an EFS of 44%, with an average of 419 days, CI 95% (325–513), vs those of the normal gene expression group with 55.8% and an average of 744 days, CI 95% (656–831), finding significant differences (p = 0.026) (Figure 3C).

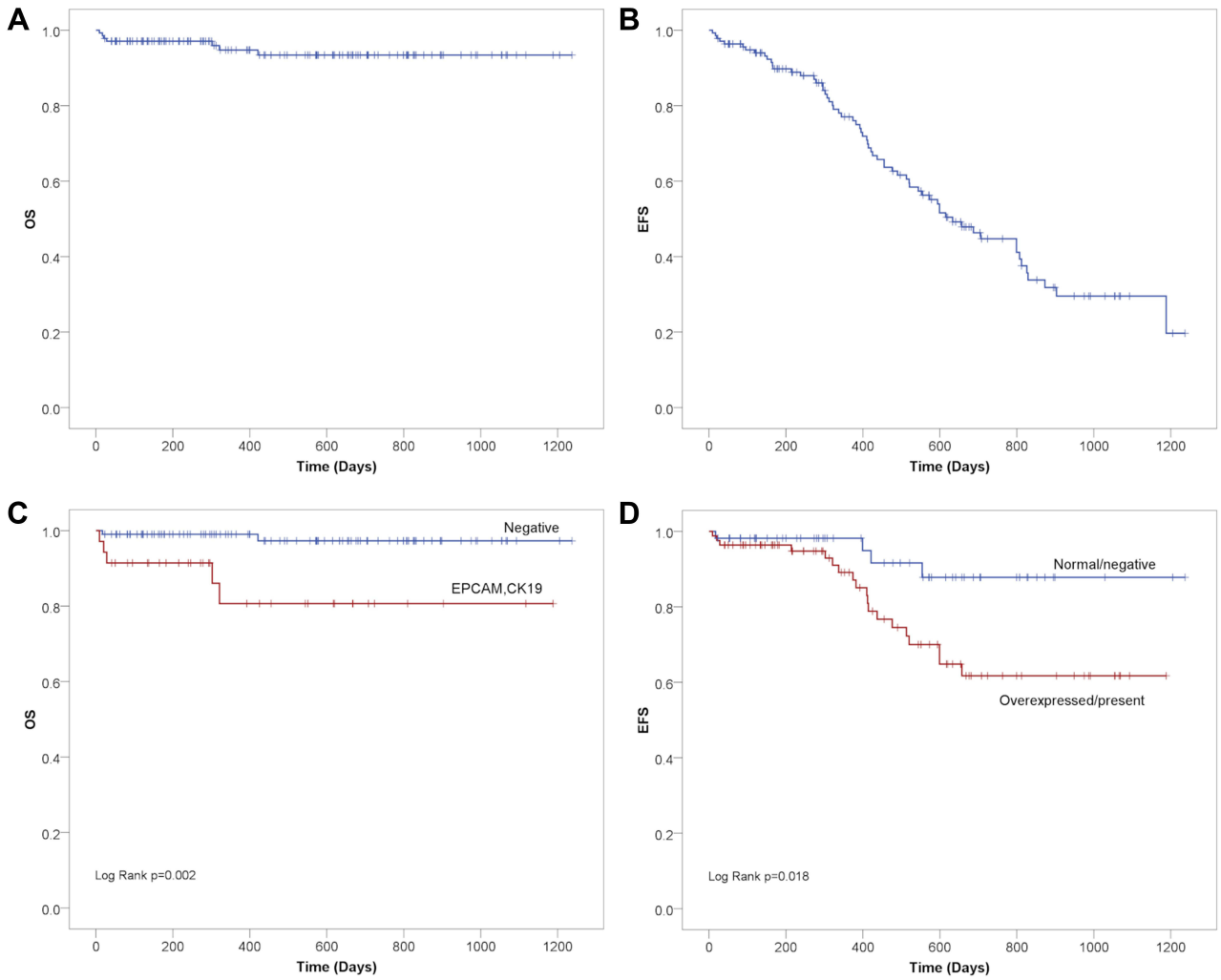

Figure 2 Overall survival and event-free survival of DLBCL patients (N=138). (**A**) General OS of DLBCL patients (94.5%). (**B**) General EFS of DLBCL patients (53.6%). (**C**) OS of DLBCL patients with presence of *EpCAM* or *CK19* (85.7%) and negative (98.1%). (**D**) EFS in DLBCL patients with overexpressed/present (70.7%) and normal/negative (91.7%) biomarkers.

Analyzing the non-GCB histological variant, it showed a similar expression profile, where genes *BCL2* (p = 0.002) (Figure 4A), *TWIST1* (p = 0.008) (Figure 4B) and *VEGFR1* (p = 0.022) (Figure 4C) reduce EFS. In addition to this, the *BCL6* gene also showed significance (p = 0.022) (Figure 4D).

Figure 5 shows the impact of the expression of the 4 genes described, where it is observed that the EFS of patients with expression of one of the 4 biomarkers is lower (32%), with an average of 370 days, CI 95% (282–458), in regard to negative patients (66.7%) with an average of 815 days, CI 95% (638–992) (p = 0.001).

As for risk factors, we found that the presence of *EpCAM* and *CK19* genes give a high risk for worse survival (OR 0.82, CI 0.14–1.50, p=0.008, and OR 0.80 CI, p = 0.012, respectively). The presence of *TWIST1* (OR 0.65, CI 0.21–1.08, p = 0.002) and *BCL2* (OR 0.55, CI 0.17–0.93, p = 0.003) showed a higher risk for EFS (Figure 6).

Discussion

Currently, more than half of patients with DLBCL can achieve remission with the current R-CHOP regimen, which represents one of the successes of recent cancer therapy. However, approximately 30 to 40% of patients will develop a recurrent or refractory disease that remains one of the main causes of morbidity and mortality in patients who have this pathology.[18] The monitoring of the disease during the progression of DLBCL to a disseminated state is usually identified with image studies such as PET; however, its access is limited and expensive, so the need to have molecular-specific,

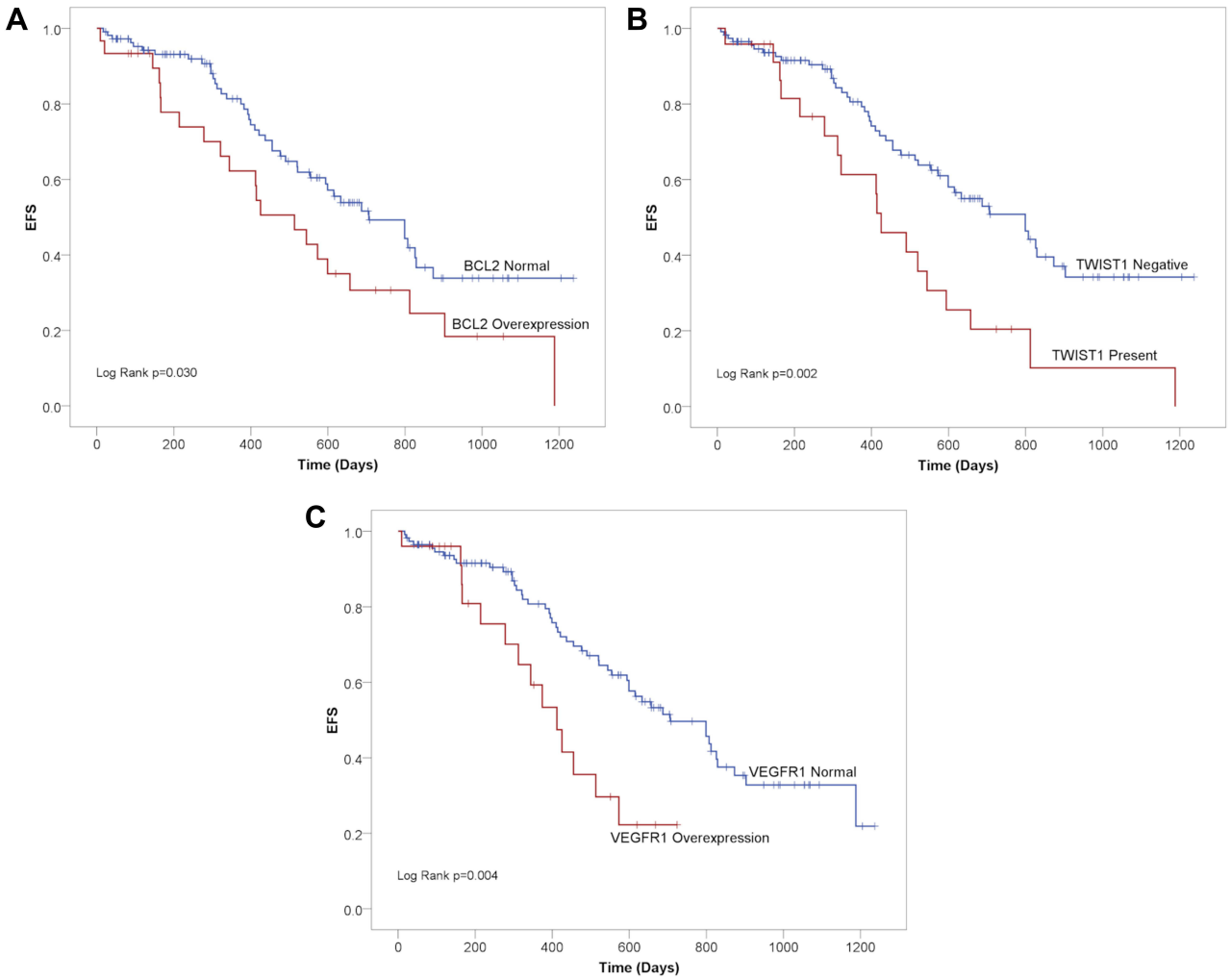

Figure 3 EFS in DLBCL patients with biomarkers expression (**A**) *BCL2* normal (60.3%) and overexpression (30%). (**B**) *TWIST1* negative (59.6%) and expression (25%), and (**C**) *VEGFR1* normal (58.8%) and overexpression (44%).

sensitive and low-cost strategies arises. The search for biomarkers in CTCs of liquid biopsies of peripheral blood might cover this need and will help us to predict, prevent and customize therapeutic strategies that will help improve the quality of life of patients.[19–21]

In this work, we analyzed the expression profiles of the biomarkers in the CTCs through RT-qPCR, and we found overexpression of *BCL2, BCL6, VEGFR1, αSMA* and *ABCB1*, and presence of *EpCAM, CK19, MAGE-A4, SNAIL* and *TWIST1*, involved in mechanisms that lead to oncogenic events and metastasis.[22,23] There are few studies using liquid biopsies derived from patients with DLBCL, one of them evaluated the expression of the mRNAs for the search for genes (*C-MYC, BCL-XL, BCL-6, NF-κB, PTEN* and *AKT*) in exosomes obtained from plasma of liquid biopsies for the monitoring and clinical evolution, their result showed that overexpression of the mRNA of the *BCL6* gene is associated with worse prognosis.[24] Thus, it is demonstrated that the search for these circulating biomarkers in DLBCL patients represents an important prognostic tool.

The Kaplan–Meier test showed that the OS of patients with expression of *EpCAM* and *CK19* was poor compared to those who did not express them (98.1% vs 85.7%, p = 0.003). The above is consistent with the study by De Wit et al in 2015, where, when analyzing CTCs of metastatic lung cancer samples, they found that patients with CTCs who had *EpCAM* and cytokeratin expression showed a decreased OS compared to patients who had no presence of these genes (p = 0.007).[25,26] In another study, 871 prostate cancer samples were analyzed, and it was found that *EpCAM* expression

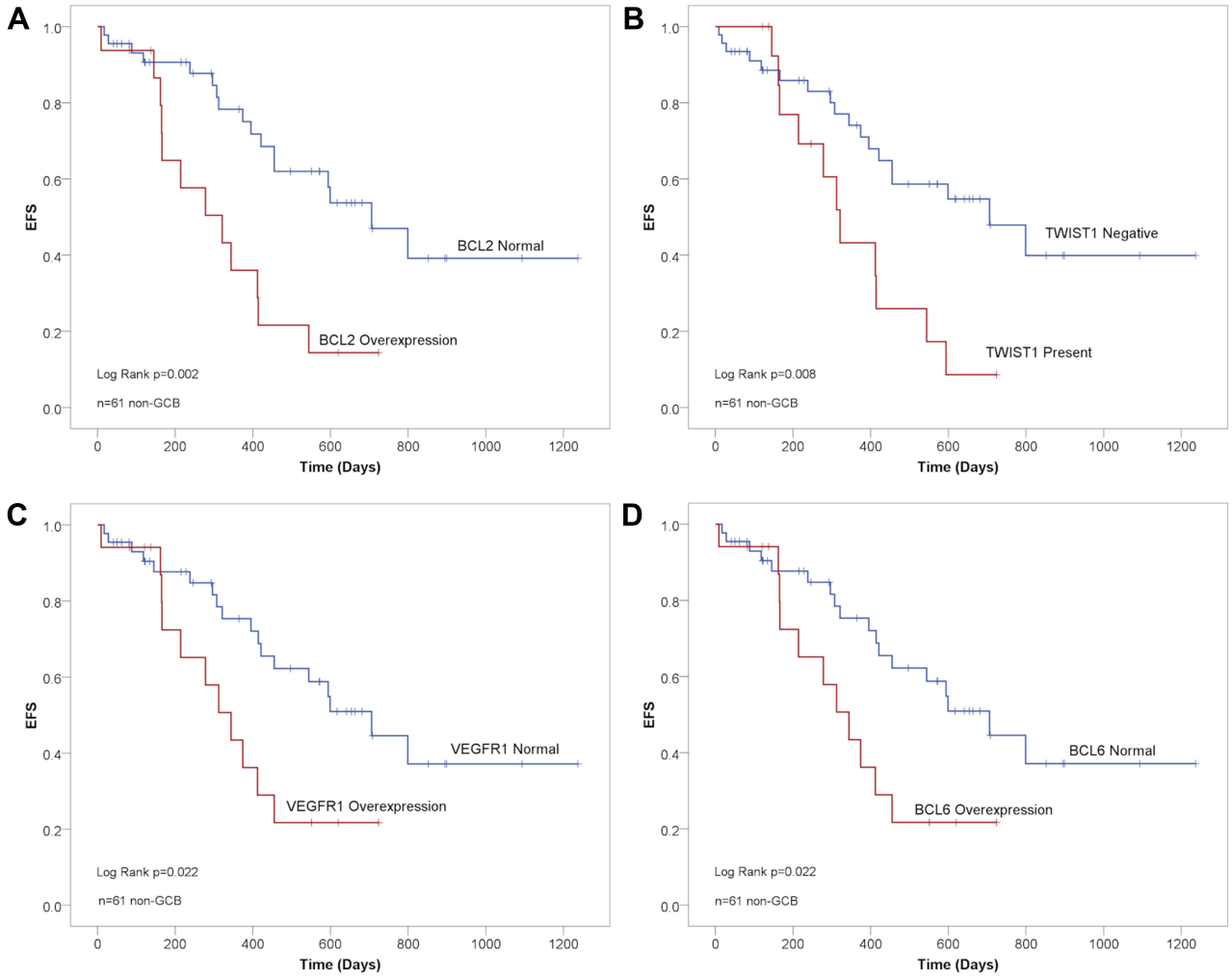

Figure 4 EFS in non-GCB DLBCL patients with biomarkers expression (**A**) *BCL2* normal (62.2%) and overexpression (25%). (**B**) *TWIST1* negative (60.9%) and expression (26.7%) (**C**) *VEGFR1* normal (59.1%) and overexpression (35.3%) and (**D**) *BCL6* normal (59.1%) and overexpression (35.3%).

was associated with worse OS.[13] Similarly, another study with 137 breast cancer patients concluded that *EpCAM* expression predicted a bad prognosis with respect to OS and EFS ($p = 0.015$, $p = 0.006$, respectively).[27]

When evaluating the impact that overexpressing biomarkers have in the EFS, we found that *BCL2, VEGFR1* and the presence of *TWIST1* were associated with a reduced EFS. Although in most of the studies in which these biomarkers are evaluated by immunohistochemistry, and lesser studies through RT-qPCR, the expression of *BCL2* shows results similar to those reported in the meta-analysis carried out by Li et al in 2018, where they concluded that *BCL2* overexpression is associated with unfavorable prognosis of DLBCL patients treated with R-CHOP.[28]

Also, it agrees with what was reported by Roh et al in 2020, where 332 patients with DLBCL treated with R-CHOP were analyzed, and an association was found between the high expression of *BCL2* and an unfavorable clinical behavior ($p = 0.01$), represented by resistance to the first line of treatment and greater mortality than in patients with low expression.[29]

The expression of BCL6 as a prognostic factor remains controversial, some studies describe its overexpression in primary tumors as a marker of poor prognosis,[19,30] others describe it as a good prognosis marker[20,31] It is important to highlight that this biomarker has not been studied as part of the characterization of CTCs to date in any type of cancer; but our study group found association between *BCL6* expression on CTCs and unfavorable clinical behavior (relapse and refractory). This phenomenon may be due to the fact that *BCL6* contributes directly to the repression of the tumor suppressor genes *P53, P21* and the pro-apoptotic protein PUMA, thus preventing the arrest of the cell cycle and

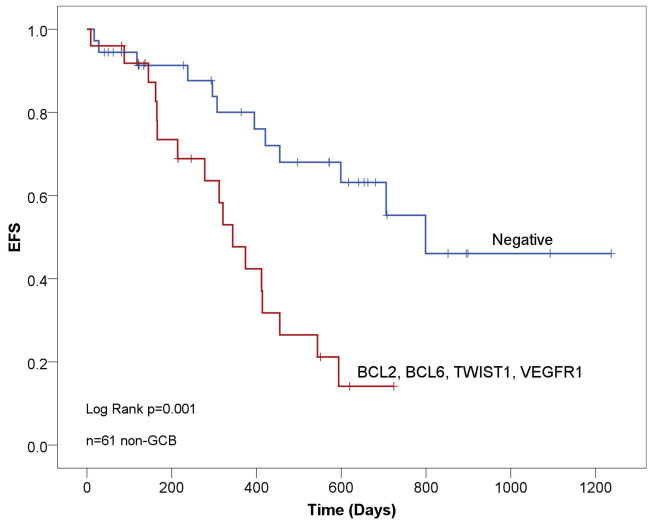

Figure 5 EFS in non-GCB DLBCL patients with *BCL2, BCL6, TWIST1* and *VEGFR1* overexpression (32.0%) and without (66.7%).

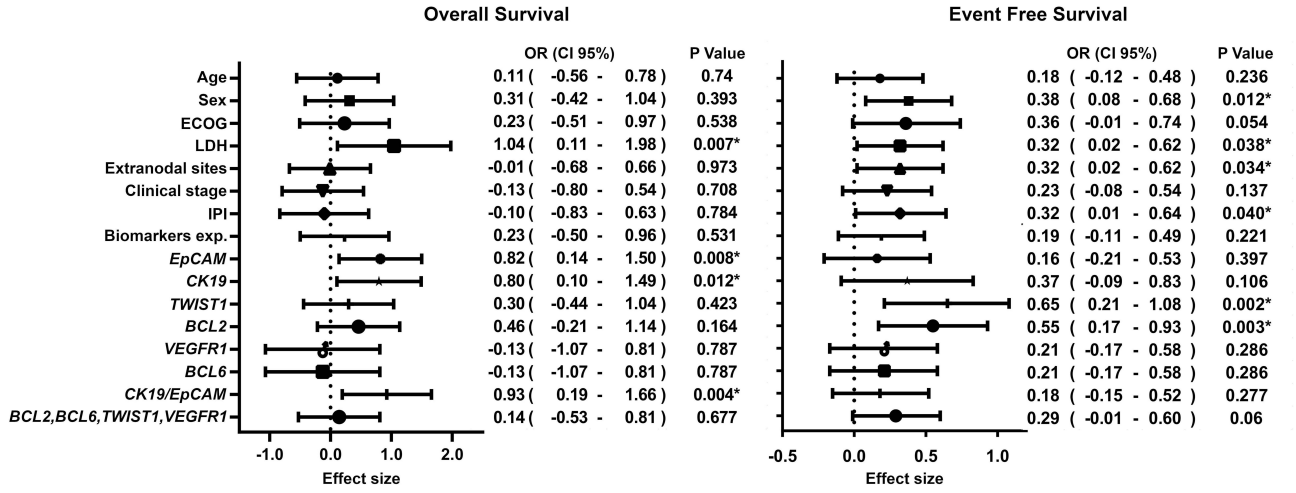

Figure 6 Odds ratio association of clinicopathological features/CTC gene expression and OS/EFS in DLBCL patients.

inhibiting apoptosis in response to the damage of DNA, that it should be caused by chemotherapeutic drugs in tumor cells, thus favoring a more aggressive pathology course.[32–34]

Like *BCL6, VEGFR1* has not been characterized in CTCs in any type of cancer, and the overexpression of this angiogenic receptor promotes tumor vascularization and favors the consequent generation of metastases; for that reason, its overexpression in cervical cancer has been described as a low survival predictor.[35] Our research group found association with a diminished EFS compared against patients with normal levels of this (55.8% vs 44%, p = 0.004). Finally, *TWIST1* expression is involved in invasion and metastasis processes, as well as the induction of angiogenesis, proliferation, and resistance to treatment mediated by the expression of ABC (ABC, ATP Binding Cassette) drug transporters.[36] In our study, the *TWIST1* gene impacts in a worse EFS; thus, the molecular identification in liquid biopsies will allow the clinician to have a tool that will help the therapeutic strategy.[37]

When we analyzed the non-GCB histological subtype, the overexpression of the *BCL2, BCL6, TWIST1* and *VEGFR1* genes confers a poor EFS, this is explained because these genes are related to inhibition of apoptosis, drug resistance, angiogenesis and metastasis, so this group has a poor survival and worse prognosis. The analyzed molecular panel would help strengthen the subclassification of this highest risk group.[38]

In the odds-ratio risk analysis (OR), results similar to those reported in the literature were obtained, observing that patients with *EpCAM*[39,40] and *CK19*[41,42] had greater death risk. Patients with overexpression of *BCL2*[43] and presence of *TWIST1*[15,37] had a higher risk of reduced EFS.

It is important to mention that the greatest limitation of our research work is the follow-up time of the patients analyzed, since compared to other survival studies in DLBCL it is shorter. However, based on the stated objective of demonstrating the value of CTCs in refractoriness and early relapse, the follow-up time was sufficient to obtain the result of the dependent variables.

Conclusion

In conclusion, our findings suggest that the detection of the previously mentioned genes in CTCs from liquid biopsies has great potential to establish an accurate molecular prognosis in patients with DLBCL. By identifying the overexpression of these biomarkers, not only at the time of diagnosis, but during the disease and even in patient surveillance, it could be useful to complement current treatment strategies. Its full implementation in the clinical environment still requires some work of validation and standardization, but the evidence shown in this and other studies indicates that in the near future, it will be a standard for prognosis in DLBCL patients.

Data Sharing Statement

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Acknowledgments

Rafael Cerón is grateful to CONACYT for a scholarship (CVU #744744) and to Posgrado en Ciencias Biológicas, Universidad Nacional Autónoma de México for academic formation. This study was supported by AMGEN (20187475) and Hospital General de México (DI/19/103/03/006 and DI/16/103/03/035).

Disclosure

The authors report no conflicts of interest in this work.

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