

Undifferentiated immunophenotypes and not expression of BCR-ABL can be associated in adult Mestizo Mexican patients with ALL

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ABSTRACT

Introduction. The BCR-ABL t(9;22)(q34;q11) translocation has been identified as a risk factor in *de novo* acute lymphoblastic leukemia (ALL), but there are other factors that may influence survival in patients not expressing this translocation. **Objective.** To associate expression and non-expression of BCR-ABL with immunophenotype and other clinical features in adult patients with ALL from a Mexican mestizo population. **Material and methods.** Peripheral blood samples from 35 adult patients with *de novo* ALL were used to detect BCR-ABL by reverse transcriptase polymerase chain reaction (RT-PCR) as well as immunophenotype by flow cytometry. **Results.** In the group of BCR-ABL negative patients (74.28%) two subgroups were identified with the immature immunophenotypes CD34+/CD33+ and/or CD13+, and CD10-/CD34+. In the group of BCR-ABL positive patients (25.72%) leukemic blast cells with a more differentiated immunophenotype compared to the BCR-ABL negative group were found. As regards clinical and biological characteristics, we found survival in months to be very similar and a tendency to high initial leukocyte counts in both groups. **Conclusions.** This is the first study conducted on a Mexican mestizo population to report that BCR-ABL negative patients can present a high frequency of undifferentiated immunophenotypes and must therefore be considered as vulnerable as BCR-ABL positive patients.

Key words. BCR-ABL. ALL. Immunophenotype.

Inmunofenotipos inmaduros y la no expresión de BCR-ABL pueden estar asociados en pacientes mexicanos adultos con LLA

RESUMEN

Introducción. La translocación t (9:22) (q34:q11)/BCR-ABL ha sido identificada como un factor de riesgo en la leucemia linfoblástica aguda (LLA) *de novo*. Sin embargo, hay otros factores que pueden influir en la supervivencia de los pacientes que no expresan dicha translocación. **Objetivo.** Relacionar la expresión y no expresión de BCR-ABL con el inmunofenotipo y otras características clínicas en pacientes adultos con LLA de población mestiza mexicana. **Material y métodos.** Se analizaron muestras de sangre periférica de 35 pacientes adultos con LLA *de novo*; a partir de éstas se realizó la detección de BCR-ABL por RT-PCR y el inmunofenotipo por citometría de flujo. **Resultados.** En el grupo de pacientes BCR-ABL negativos (74.28%) se distinguieron dos subgrupos con inmunofenotipos inmaduros CD34+/CD33+ y/o CD13+ y CD10-/CD34+. En el grupo de pacientes BCR-ABL positivo (25.72 %) se encontraron blastos leucémicos con inmunofenotipo más diferenciado respecto al grupo BCR-ABL negativo. En cuanto a las características clínico-biológicas estudiadas se observó supervivencia en meses muy semejante y tendencia a presentar cuentas altas de leucocitos iniciales en ambos grupos. **Conclusiones.** Éste es el primer estudio realizado en población mestiza mexicana que muestra que los pacientes BCR-ABL negativos pueden presentar inmunofenotipos indiferenciados; por lo cual deben ser considerados como un grupo tan vulnerable como los pacientes BCR-ABL positivo.

Palabras clave. BCR-ABL. Leucemia linfoblástica aguda. Inmunofenotipo.

INTRODUCTION

Acute lymphoblastic leukemia (ALL) is a heterogeneous group of lymphoid disorders of uncertain response to therapy. Diagnostic progress so far includes differentiation of subtypes of ALL, in which the biological factors taken into account include the presence of chromosomal aberrations such as translocations; morphologic and immunophenotypic characteristics of leukemic blasts; and clinical factors guiding classification of the disease into high- and low-risk groups in order to be able to apply individualized therapies to each patient.¹ Immunophenotyping permits the classification of leukemic blasts into T-, B-, or biphenotypic lineages based on degree of maturation and expression of specific lymphoid and/or myeloid markers.²⁻⁵ The co-expression of CD34 with B- or T-lineage markers (CD20 or CD19; CD5 or CD7, respectively) is common in progenitor B and T cells.⁵ These lymphoid progenitor cells have fully lost the capacity to differentiate into other lineages and do not therefore express myeloid markers.⁶ The co-expression of CD34 and myeloid antigens (CD33/CD13) in lymphoblasts indicates that these blast cells come from multilineage leukemia stem cells.⁷⁻⁹ The latter cells are able to induce their own proliferation by autocrine secretion of growth factors such as stem cell factor (SCF) and interleukin-3 (IL-3).¹⁰ Likewise, when these cells initiate proliferation, they show shorter division cycles than non-leukemic stem cells, which contributes to a higher proliferation rate. This is evidenced by an increase in the number of leukemic cells present in the body.¹¹ The variant of ALL expressing myeloid markers has been associated with high leukocyte counts and a high proportion of circulating blasts as well as low survival.¹²⁻¹⁵

The t(9;22)(q34;q11) translocation is an abnormal genetic rearrangement occurring in 20 to 30% of adult patients with ALL, with an incidence of > 50% in individuals age 50 or older.¹⁶⁻¹⁸ This translocation results from the junction of 3' sequences of the c-ABL (9q; 34) tyrosine kinase proto-oncogene on 5' sequences of the BCR (22q; 11) gene, inducing expression of a fusion protein with two isoforms: BCR-ABL p190 and BCR-ABL p210.^{1,18-20} Both isoforms have unregulated tyrosine kinase activity and several domains that recruit diverse signaling proteins which contribute among other effects to the emergence of neoplastic cells with high proliferation rates, interruption of normal apoptosis, and rearrangement of the cytoskeleton,²¹⁻²⁴ so that expression of these fusion proteins is associated with a poor prognosis.^{25,26} Similarly, a high incidence of expression of myeloid markers has been observed in

cells expressing the BCR-ABL translocation.¹³ Specifically in ALL, expression of the markers CD33 and CD13 has been reported in 27 to 50% of BCR-ABL positive cells and 14 to 25% of BCR-ABL negative cells.^{16,27-29} The marker CD10 (common acute lymphoblastic leukemia antigen, cALLA) has been associated with BCR-ABL positive patients, while non-expression of this marker (CD10-) has been linked to BCR-ABL negative patients showing also the MLL-AF4 (11q23) translocation. It is worth noting that these patients showed low survival rates.³⁰

In our mestizo population, adult patients with *de novo* ALL were in general observed to have a poor survival prognosis and high relapse rates after a complete remission.

OBJECTIVE

To associate expression and non-expression of BCR-ABL with lineage, degree of maturation of leukemic lymphoblasts, initial leukocyte count, and survival in order to identify in these patients poor prognosis factors other than BCR-ABL; such as degree of differentiation and high proliferation rate, which may favor lymphoblast development and persistence.

MATERIAL AND METHODS

Patient samples

Peripheral blood and bone marrow was obtained from 35 patients with *de novo* ALL. The diagnosis was established by analyzing clinical signs, morphology, cytochemistry and immunophenotype in bone marrow aspirate using the criteria of the hospital center from which the samples came. The following hospital centers took part in this study: National Medical Center La Raza (Mexican Social Security Institute, IMSS) and the General Hospital of Mexico., both considered hub centers for treatment of this disease in Mexico. Before starting study, all patients signed an informed consent authorization. The procedures done in this protocol were in accordance with the Helsinki Declaration of 1975. The protocol was approved by the Ethics Committee of the participating hospitals.

Isolation of mononuclear cells and RNA extraction

Mononuclear cells were isolated from peripheral blood or bone marrow by density gradient centrifugation with Ficoll-Paque PLUS (GE Healthcare).

RNA was extracted from cells using a QIAshredder spin column kit (Qiagen) according to manufacturer instructions, in order to subsequently detect transcripts of BCR-ABL p210 and p190 isoforms by reverse transcriptase polymerase chain reaction (RT-PCR).

RT-PCR

RNA was adjusted to 1 μ g and retrotranscribed with a QuantiTect Reverse Transcription kit (Qiagen) using a Mastercycler Personal thermocycler (Eppendorf). The oligonucleotide primers for BCR-ABL major and minor translocation regions (p210 and p190, respectively) were obtained and amplified according to the methodology described by van Dongen, *et al.*¹⁸ Amplification of the actin gene was done using the following oligonucleotides:

- Forward: 5'-GAGCGGTTCCGCTGCCCTGA-3'.
- Reverse: 5'-GCCGCCGATCCACACGGAGT-3'.

PCR products of BCR-ABL and actin were analyzed in 0.9% agarose gel using ethidium bromide.

Determination of immunophenotype

Bone marrow samples were taken from patients before treatment and processed immediately after collection in the same laboratory by the same technician. The total number of mononucleated cells was obtained from the blood count. Cell number and lymphoblast cell population were obtained by flow cytometry using FACS Calibur (Becton Dickinson, San Jose CA, USA). For staining, aliquots of whole blood containing up to 10⁶ leukocytes were incubated with the adequate amount of each monoclonal antibody for 15 min at room temperature. Red blood cell lysis was performed with FACS Lysing Solution (Becton Dickinson). Cells were washed twice with phosphate buffered saline (PBS) after which acquisition was made of 10,000 events selected from two-parameter dot plots using a base 10 logarithmic scale for both the x-axis (FL1, fluorescein isothiocyanate = FITC) and y-axis (FL2, phycoerythrin = PE). The total cell population in the vial marked isotype control was acquired. The population of blasts was identified with the use of the antibody CD34 and subsequently by size and granularity within the total population. Results for the selected cellular region were expressed as percentages. Adequate isotype controls were used. The

criteria established by the European Group for the Immunological Characterization of Leukemia (EGIL)³¹ were taken into consideration, assuming that the criterion for positivity of surface marker expression was at least 25%, and at least 10% for intracellular antigens. B-lineage expression was defined by positivity for the markers, CD79a, CD19, CD20 and CD22; T-lineage expression by positivity for the markers CD5 or CD7; and biphenotypic lineage expression by positivity for the myeloid markers CD13 and/or CD33 along with the markers CD79a CD19, CD20 and CD22 for B cells and CD5/CD7 for T cells. The origin of blast cells was determined in each patient by positivity for the markers CD34 and CD10 as well as expression of the myeloid markers CD33/CD13.

Statistical analyses

χ^2 was used to compare clinical parameters between BCR-ABL positive and BCR-ABL negative patients, as well as the different BCR-ABL transcripts. Survival was defined as the period of time from diagnosis to conclusion of the study or death. Survival time was estimated by the Kaplan-Meier method and compared by log-rank test. Differences between means were obtained by the Wilcoxon test. Statistical analyses were performed with SPSS for Windows v.10.0.

RESULTS

- **BCR-ABL fusion transcripts.** Of the 35 patients studied 25.72% (9/35) expressed the translocation (BCR-ABL positive) and 74.28% (n = 26) did not (BCR-ABL negative) (Table 1). In the group of BCR-ABL positive patients, BCR-ABL p190 transcripts were detected in 4/9 patients (44.44%), four more patients (44.44%) expressed BCR-ABL p210 transcripts, and one (11.11%) co-expressed BCR-ABL p190 and p210 transcripts (data not shown).
- **Immunophenotypes of BCR-ABL positive and BCR-ABL negative groups.** Immunophenotyping of leukemic blast cells was performed on all patients studied. In the group of BCR-ABL positive patients, 7/9 patients (77.8%) were B-lineage and two (22.2%) were T-lineage. In the BCR-ABL negative group, 20/26 patients (76.9%) were B-lineage, one (3.8%) was T-lineage, and five (19.2%) were biphenotypic. Significant difference was found between both groups alone for the lineage B (Table 1). As regards expression of markers of cellular differentiation, in the

Table 1. Blast cell lineage and immunophenotype in positive and negative BCR-ABL patients.

	BCR-ABL positive, n = 9 (%)	BCR-ABL negative, n = 26 (%)
• Immunophenotype		
ALL B	7 (77.8)*	20 (76.9)*
ALL T	2 (22.2)	1 (3.8)
ALL T & B	0 (0)	5 (19.2)
• Cell markers		
CD34	6 (66.7)*	6 (23.1)*
CD34+/CD33+ and/or CD13+/CD10+	0 (0)*	6 (23.1)*
CD10+ (CALLA)	2 (33.3)	14 (53.84)
CD10- (CALLA)	7 (77.8)	12 (46.2)

* $p < 0.05$. The χ^2 test was used to compare the parameters of BCR-ABL-positive and negative patients.

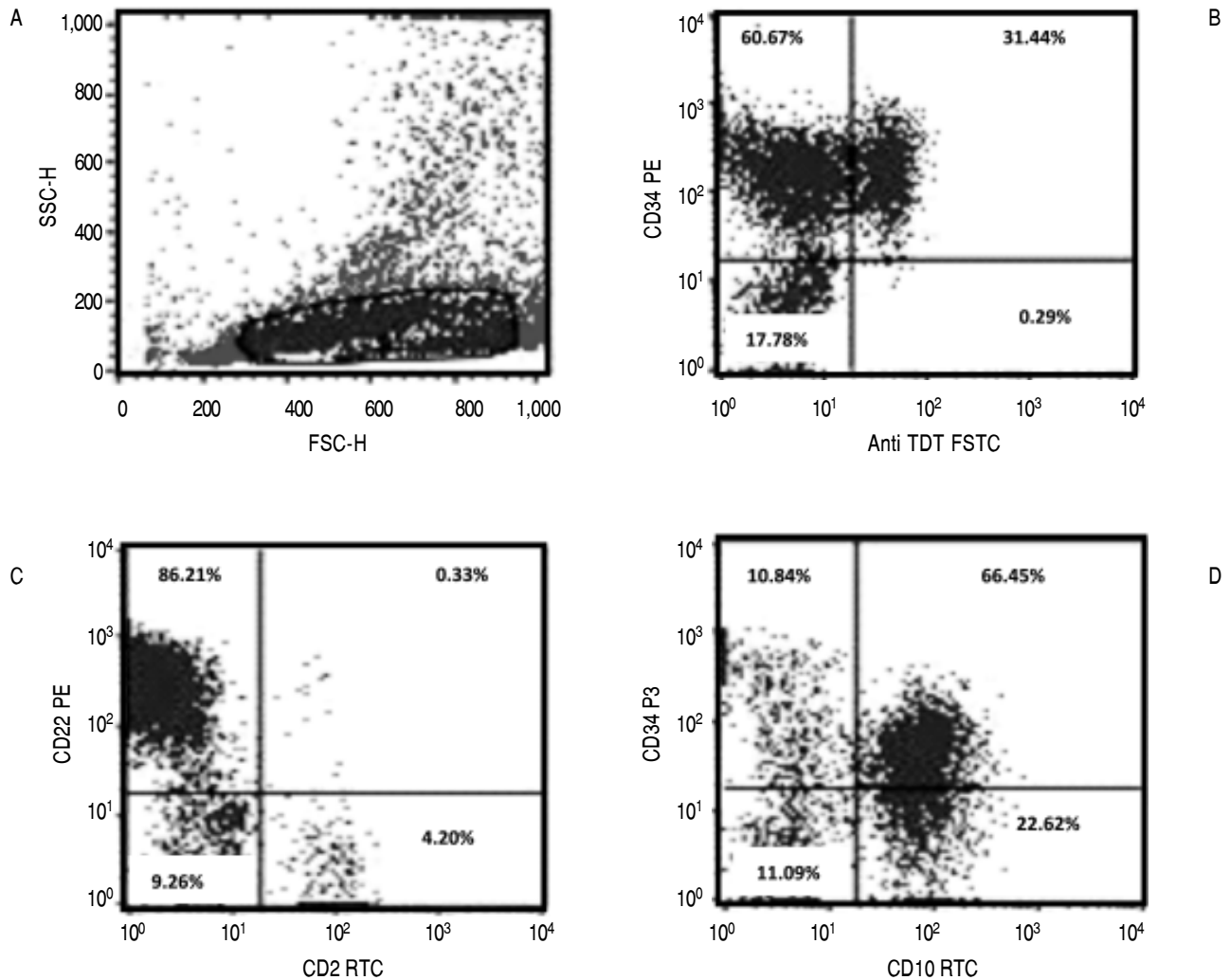


Figure 1. Immunophenotype profile of bone marrow from ALL patients. Analysis was performed on a FACScalibur flow cytometer, gating on the blast cell population; all samples contained more than 60% of blast. Double labeling was carried out with FITC (fluorescein) and PE (phycoerythrin) conjugated monoclonal antibodies. Nuclear and cytoplasmic staining was carried out by flow cytometry after fixation and permeabilization of the cells. A. Lymphoblast populations were selected from the total cell sample. B. 92.11% of blast cells expressed CD34. C. 86.21% of blast cells expressed B-lymphoid marker CD22. D. 66.45% of blast cells co-expressed the myeloid antigen CD13 and CD10.

Table 2. Comparison of clinical and biological characteristics in BCR-ABL positive patients and BCR-ABL negative patients with immature immunophenotype.

Clinical features	BCR-ABL positive, n = 9 (%)	BCR-ABL negative		
		n = 26 (%)	CD34+/CD33+ and/or CD13+, n = 6 (%)	CD34+ / CD10-, n = 10 (%)
• Age (years)				
< 50	5 (55.6)	22 (84.6)	6 (100)	8 (80)
> 50	4 (44.4)*	4 (15.4)*	0 (0)	2 (20)
• WBC (μ L)				
< 30,000	5 (55.6)	15 (57.7)	0 (0)	10 (100)*
> 30,000	4 (44.4)	11 (42.3)	6 (100)*	0 (0)
• Survival				
Median	10 months [†]	14 months	16 months [†]	4 months [†]
Range	5.94-17.15	9.83-20.98	4-24	0.1-7

*p < 0.05. The χ^2 test was used to compare the parameters of BCR-ABL-negative CD34+/CD33+ and/or CD13+ and CD10-/CD34+ groups of patients. [†]p < 0.001. Median values were compared by the 2-sided Wilcoxon Mann-Whitney test to compare the survival of BCR-ABL positive and negative patients.

BCR-ABL positive group 6/9 patients (66.7%) expressed CD34 and 2/9 (33.3%) expressed CD10. The immunophenotype CD10- was found in seven patients (77.8%) in the BCR-ABL positive group, one patient did not express any of the markers evaluated (data not shown), and none of the nine BCR-ABL positive patients expressed the markers CD33 and/or CD13. In the BCR-ABL negative group, 6/26 patients (23.1%) expressed CD34, these patients also co-expressed CD34/CD33 and/or CD13; 14/26 (53.84%) expressed CD10, while the immunophenotype CD10- was found in 12 (46.2%); one example of immunophenotyping process is shown in figure 1. Five patients expressed the markers CD34+/CD10+ (19.23%) and a further five (19.23%) did not express any of the markers evaluated (data not shown). CD34 expression was greater in the BCR-ABL positive group compared to the BCR-ABL negative group (66.7% vs. 23.1%, $p \leq 0.05$). Likewise, the co-expression of CD34/CD33 and/or CD13 was statistically significant only for the BCR-ABL negative group (0 vs. 23.1%, $p < 0.05$) (Table 1).

- **Clinical and biological characteristics of BCR-ABL positive and BCR-ABL negative patients.** In the BCR-ABL positive group, 5/9 patients (55.6%) were under 50 years of age and had leukocyte counts < 30,000/ μ L, while 4/9 (44.4%) were over 50 years of age and had initial leukocyte counts > 30,000/ μ L. On the other hand, in the BCR-ABL negative group, 22/26 patients (84.6%) were under 50 years of age and only 4/26 (15.4%) were over 50 years, while 15/26 patients (57.7%) had initial leukocyte counts

< 30,000/ μ L and 11/26 (42.3%) had leukocyte counts > 30,000/ μ L. A significant age difference ($p < 0.05$) was found: BCR-ABL expression occurred more frequently in patients over 50 years old. Initial leukocyte count did not differ significantly between the BCR-ABL positive group and the BCR-ABL negative group (Table 2).

- **Clinical follow-up of patients was performed for two years after diagnosis.** It is worth noting that all patients died with active ALL. No significant difference was found when mean survival in the BCR-ABL positive group (10 months) and BCR-ABL negative group (14 months) was compared. On the other hand, in the BCR-ABL negative group more patients showed immature immunophenotypes such as CD34/CD33 and/or CD13 (6 patients) and CD10-/CD34+. ¹⁰ No significant difference in relation to age was found between these two subgroups, but the subgroup with the CD34+/CD33+ and/or CD13+ immunophenotype had leukocyte counts > 30,000/ μ L while the subgroup with the CD10-/CD34+ immunophenotype had counts < 30,000/ μ L ($p < 0.05$). In comparing the median overall survival of these groups a significant difference was found (16 vs. 4 months, $p < 0.001$), even when survival in both groups was compared to survival in the BCR-ABL positive group (10 months) (Table 2).

DISCUSSION

This study is the first to report that patients with ALL BCR-ABL negative can present a high frequen-

cy of immature biphenotypic CD34+/CD33+ and/or CD13+ immunophenotypes compared to BCR-ABL positive patients. This suggests that the blast cells of patients represent lymphoid progenitor cells characterized by their inability to differentiate into other lineages. In BCR-ABL negative Caucasian patients expression of the markers CD33 and CD13 has been reported;³⁰ this is consistent with present results. However, this frequency is not higher than the frequency obtained in BCR-ABL positive Caucasian patients. In our study, these markers were expressed only in BCR-ABL negative patients, indicating that the frequency and expression of immature immunophenotypes found in our study is unique to the BCR-ABL negative group, this has not so far been reported.

CD10 expression has been associated primarily with BCR-ABL positive patients.²⁸ Gleißner, *et al.*, in 2002,¹⁶ reported that the association of BCR-ABL positive patients with the immunophenotype CD10+ showed a frequency of 37%. In the present study, no significant difference was found in CD10 expression between the BCR-ABL positive and BCR-ABL negative groups, but > 50% of BCR-ABL negative patients express this marker, therefore, there is a tendency for our BCR-ABL negative patients to express CD10. On the other hand, Gleissner, in 2005,³⁰ found that 56 patients with the immunophenotype CD10- were BCR-ABL negative that also expressed the MLL-AF4 (11q23) translocation, all of this associated with low survival. Our study also found no significant difference between BCR-ABL positive and BCR-ABL negative groups with regard to non-expression of CD10. However, the number of patients with this immunophenotype was greater in the BCR-ABL negative group (12 vs. 7) and this group of BCR-ABL negative patients with CD34+/CD10- was also the one with the lowest survival, which is consistent with previous reports by Gleissner, *et al.*³⁰

In the BCR-ABL negative group we found that five patients (19.23%) did not express the markers CD34+/CD33+ and/or CD13+ and five more (19.23%) expressed CD34+/CD10+. This indicates that the lymphoblasts of these ten patients were more differentiated. In BCR-ABL negative Caucasian patients a frequency of ~70% differentiated immunophenotypes has been reported; this is higher than the frequency found in our patients (38.46%). These results may indicate that BCR-ABL negative patients from our population tend to show a higher frequency of immature immunophenotypes.

With respect to blast cell lineage, we found that type B predominated in both groups of patients;

which agrees with previous reports.^{1,3,32} BCR-ABL negative patients with the CD34+/CD33+ and/or CD13+ immunophenotype had leukocyte counts > 30,000/ μ L, suggesting a high proliferation rate in this subgroup. However, BCR-ABL negative patients with the immunophenotype CD34+/CD10- had leukocyte counts < 30,000/ μ L, suggesting that the cells of these patients probably show effects other than proliferation which contribute to development of ALL. One possibility may be inhibition of apoptosis, reported to occur precisely in CD10- blasts;³³ this might explain the low leukocyte count in this group of patients.

The mean age of BCR-ABL negative patients was under 50 years, while mean age in the BCR-ABL positive group was over 50 years. This is similar to findings in Caucasian populations.^{3,34} Follow-up of patients was conducted for two years, taking into account that survival in the BCR-ABL negative group ranged from 4 to 16 months, similar on average to survival in the BCR-ABL positive group (10 months). This is similar to the mean survival in Caucasian populations (11 months).³⁰ These results show that BCR-ABL positive patients in our population are similar in age and survival to above mentioned populations. BCR-ABL negative Caucasian patients with ALL are reported to be less than 50 years old.¹⁷ This is consistent with findings in our population for this same group of patients, but not as regards survival which has been reported to be higher in BCR-ABL negative patients than BCR-ABL positive patients.³⁵ As regards survival, we may therefore say that a larger number of BCR-ABL negative patients with low survival were found in our population compared to Caucasian populations since, as stated earlier, a more immature immunophenotype predominated in our patients. In the ten BCR-ABL negative patients with a more differentiated immunophenotype, survival was found to be similar to survival in patients with the biphenotypic CD34+/CD33+ and/or CD13+ immunophenotype (14 months) and higher than that in patients with the immature immunophenotype CD34+/CD10- (4 months). This suggests that the less mature the immunophenotype is, the more influence it has on patient survival.

This observation is consistent with previous reports regarding the fact that absence of CD10 has been associated with poor survival.

It is important to note that BCR-ABL negative patients in the Mexican mestizo population displayed a specific immunophenotype, since most showed immature immunophenotypes such as CD34+/

CD33+ and/or CD13+ or CD10-/CD34+, and a smaller number showed more mature immunophenotypes. This makes these patients a heterogeneous group, hard to cluster into a group and with a low survival rate similar to that of BCR-ABL positive patients. It may therefore be suggested that both presence of an immature immunophenotype and BCR-ABL expression are poor prognosis factors in our population since they affect patient survival in a smaller manner.

Nevertheless of that our work includes a number seemingly reduced of patients, as others published in Asian population,^{37,38} we suggest that Mexican mestizo patients with ALL can present an association among immature immunophenotypes and not expression of BCR-ABL, low survival and a tendency to high initial leukocyte counts, which suggests a high rate of cellular proliferation. These findings suggest that in our population, the group of BCR-ABL negative patients should be considered as vulnerable as the group of BCR-ABL positive patients. Likewise, it is necessary to continue the analysis of these patients to quantify the frequency of immature immunophenotypes in Mexican mestizo patients BCR-ABL negatives.

Furthermore, it is worth noting that our results differ from those obtained in Asian populations, where a high prevalence of immature immunophenotypes has been found in BCR-ABL positive patients.³⁶⁻³⁸ Chen, *et al.*, in 2010,³⁸ propose that an immature immunophenotype may predict the presence of the BCR-ABL translocation. However, our results indicate that BCR-ABL expression in a Mexican mestizo population may not be associated with immature immunophenotypes, perhaps due to race-related intrinsic differences.

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