ORIGINAL PAPERS

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Glycogen Synthase Kinase-3β (GSK-3β) and Nuclear Factor Kappa-B (*NFKB*) in Childhood Acute Lymphoblastic Leukemia

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Abstract

Background. Acute lymphocytic leukemia (ALL) is the most common hematologic malignancy in early childhood. In children with acute lymphoblastic leukemia (ALL), the activity of glycogen synthase kinase (GSK-3β) has been associated with changes in the transcriptional activity and expression of nuclear factor kappa beta (*NFKB*) in the mononuclear cells of bone marrow.

Objectives. The aim of the study was to determine the possible role of glycogen synthase kinase 3beta (GSK-3β) and nuclear factor kappa beta (*NFKB*) as prognostic variables in pediatric patients with ALL.

Material and Methods. This was a descriptive, transversal, and observational study. Bone marrow and blood samples were obtained from 30 children with newly-diagnosed ALL, who were seen at the Hematology-Oncology Service, Hospital para el Niño (HPN), Toluca, Mexico, from 2014–2015. Anthropometric variables, clinical lab results, immunophenotype and cytogenetic abnormalities were registered. GSK-3β was evaluated through immunohistochemistry, and *NFKB* messenger RNA (mRNA) with real-time polymerase chain reaction (qPCR). The cases of ALL were classified into two groups of risk: high and habitual.

Results. Thirty patients were included in this study, with a mean age of 7.1 years (range 2–13 years). Twenty-one were male and 9 female. Employing the morphological classification, 26 patients had type L1 ALL and the remaining 4 patients had type L2 ALL. Abnormal genes were found in 7 (23.33%) patients, ETV-RUNX1 in 3, followed by TCF3-PBX1 (two), STL1-TAL1 (one), and BCR-ABL1 (one). *NFKB* relative expression levels, in comparison to the GSK-3β immunohistochemistry results of the bone marrow samples, showed significant differences between positive and negative cases (p = 0.001) and between weak-positive and negative cases (p = 0.002).

Conclusions. These results suggest that GSK-3β may be a prognostic biomarker in childhood ALL (**Adv Clin Exp Med 2016, 25, 6, 1139–1147**).

Key words: acute lymphoblastic leukemia, GSK-3β, *NFKB*, prognostic.

Acute lymphoblastic leukemia (ALL) is the most common type of cancer among children, teenagers, and young adults. In 2002 there were approximately 1720 children living with acute lymphoblastic leukemia in Mexico. every year 60–100 new cases of leukemia per 1 million inhabitants appear in Western countries. ALL is characterized by a tremendous clinical variability, prompting a continuing search for accurate disease outcome predictors. Thus, the interactions between leukemia

cells and the microenvironment of the bone marrow are determinant to generate changes in the cell signaling pathways associated with cell survival. The molecular signaling pathways associated with the hematopoiesis process are varied but deregulation in one or more points should be decisive in ALL development. Moreover, the cytogenetic pattern is an important determinant of ALL development; for example, t(1,19), t(4,11), t(12,21) and t(9,22) affect the expression of the enzymes that regulate the intracellular signaling process [1]. As we know, immunophenotype, age, race, white blood cell (WBC) count, and gender are important prognostic factors.

Glycogen synthase kinase 3 (GSK-3) is a serine/tyrosine kinase identified in skeletal muscle. To date, two isoforms of GSK-3 have been reported: the first, 51 kDa (GSK-3α), and the second, 47 kDa (GSK-3β). The activity of GSK-3 can be inhibited by Akt-mediated phosphorylation at Ser21 in GSK-3α and at Ser9 in GSK-3β. The aberrant expression of GSK-3β has been demonstrated in metabolic disorders and in hematopoietic stem cell differentiation and proliferation [2]. Several studies have been performed by means of cell culture and immunohistochemistry and have shown that GSK-3β is found significantly accumulated in the nuclei of cells in patients with ALL. Furthermore, selective inhibition of GSK-3β induces cell death mediated by downregulation of the transcriptional activity of hematopoietic nuclear factor kappa-light-chain-enhancer of activated B cells (*NFKB*). Thus, GSK-3β inhibitors significantly decreased *NFKB* expression, generating gene suppression and stimulating apoptosis *in vitro*, which suggested its being a novel, interesting target in ALL treatment [3]. Even more so, the activation process of *NFKB* in leukemic cells is associated with different micro-environmental stimuli. However, determination of the biomarkers associated with the prognosis and treatment of ALL in the last decade has been insufficient.

The *NFKB* signaling pathway has exhibited constitutive aberrant activation in various hematologic malignancies. In fact, *NFKB* plays an important role as the mediator of different processes, including immunity, inflammation, cancer promotion, and tissue regeneration [4]. Moreover, *NFKB* regulates multiple cell processes including survival, proliferation, and migration. Alterations of the *NFKB* signaling pathway have been associated with the survival of leukemic cells. In addition, selective inhibition of *NFKB* in *in vitro* studies has demonstrated the elimination of these with minimal effects on normal stem cells [5].

The principal aim of this study was to determine whether *NFKB* and GSK-3β expression in childhood ALL could be useful as prognostic variables.

Material and Methods

Patients

All children diagnosed with ALL from February 2014 to March 2015 at the Hospital para el Niño (HPN), Instituto Materno Infantil del Estado de México (IMIEM), Toluca, Mexico, were invited to participate in the study. The patients were classified into two groups by risk: a) habitual risk, and b) high risk. Patients were not included in this study if they suffered from another chronic disease. The established treatment was that recommended by the St. Jude Children's Research Hospital [6].

General Information

The clinical data was obtained from the patient's clinical database file (Histoclin v. 2.0, Palmera Sistemas, Mexico City, Mexico). All children were measured for height (m) and weight (kg). Body mass index (BMI) was calculated as weight (kg) divided by height squared (m^2).

Laboratory Analysis

Biochemical and immunophenotype studies were performed at the Clinical Laboratory, HPN, IMIEM. Immunohistochemical and molecular analyses were conducted at the Molecular Biology Laboratory, Medical Sciences Research Center (CICMED), Autonomous University of the State of Mexico (UAEMex).

Biochemical Studies

Blood samples were collected into Vacutainer™ tubes and centrifuged to separate serum from plasma to measure fasting venous serum glucose (mg/dL), uric acid (mg/dL), albumin (mg/dL), cholesterol (mg/dL) and triacylglycerols (mg/dL) (Hitachi 911, Roche Diagnostics, San Francisco, USA) and hematic biometry (KX-21N™, Sysmex, Mundelein, USA). All measurements followed standardized procedures according to the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC).

Immunophenotype

Peripheral blood and bone marrow samples were processed in all cases. Bone marrow samples were collected and stored under deep-freezing conditions (−80°C) (Revco Value® Thermo Scientific, Waltham, USA) until their use. Immunophenotype was determined by flow cytometry (Gallius, Beckman-Coulter, Indianapolis, USA).

The markers used were as follows: CD34; CD45; CD117, HLA-DR and TdT (immature cells); B-lymphoid lineage (CD10, CD19, CD20, CD22, and CD79a); T-lymphoid lineage (CD3 and CD7), myeloid lineage marker CD13, and the myeloperoxidase (MPO) marker. The chromosomal rearrangements analyzed are shown in Table 1.

Gene Expression

mRNA Extraction

Approximately 200 μL of each sample was suspended in 300 μL of ACK buffer (Lonza, San Francisco, USA) to isolate the mononuclear cells of bone marrow (MCBM). RNA was extracted from MCBM using Magna Pure LC RNA Isolation Kit III-Tissue (Roche) in the Magna Pure LC 2.0 Instrument (Roche, Penzberg, Germany). The A260/280 nm absorbance ratio was > 1.8 (quality) and total RNA concentration was calculated by determining absorbance at 260 nm with the NanoPhotometer (Implen GmbH, München, Germany).

Real-time PCR

Reverse transcription and quantitative real-time PCR (qPCR) were performed with the 7500 Fast Real Time PCR System (Applied Biosystems, Cheshire, UK), employing the Sybr Green one-step RT-PCR kit (Sigma-Aldrich, Foster City, USA), mixing 0.4 mM of each primer in a final volume of 25 μL, the final concentration of each gene was optimized with the Taguchi method. The qPCR

program included 30 min at 43°C, 2 min at 95°C; 40 cycles of 15 s at 95°C, 1 min at 60°C followed by a melting curve. The annealing temperature for all primers was 60°C. The comparative threshold cycle (CT) method was utilized to calculate fold amplification as follows: $2^{-\Delta\Delta CT}$, where $\Delta\Delta CT$ = (CT-target − CT-reference) treated-sample − (CT-target − CT-reference) calibrator-sample. Calibrator-sample refers to the expression level (×1) of the target gene normalized to the constitutive gene [7].

The primers used in the experiments were designed using the Primer Quest web tool (Integrated DNA Technologies, Inc., Los Angeles, USA) and synthesized in the Unit of Synthesis and DNA Sequencing, Institute of Biotechnology, Autonomous University of Mexico (UNAM) (Cuernavaca, Morelos, Mexico). A Basic Local Alignment Search Tool (BLAST) search was performed on primer sequences to ensure that oligos hybridized only to specific products. Sequences of the primers utilized in the analysis were as follows: glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), (NCBI: NM_002046.5), forward: 5-CTTTGGTATCGTGGAAGGACTC-3, reverse: 5-GTAGAGGCAGGGATGATGTTCT-3, and *NFKB* p65 (NCBI: NM_003998.3), forward: 5-TGGGAATCCAGTGTGTGAAG-3, reverse: 5-CACAGCATTCAGGTCGTAGT-3.

Immunohistochemistry

Slides were fixed with ethanol at 96%. Briefly, following conventional dehydration, the bone marrow was transparently embedded in slices. In a second

Table 1. Chromosomal rearrangements analyzed

1p32(ST1L-TAL1)	t(1;11)(p32;q23)(MLL-EPS15)
t(1;11)(q21;q23)(MLL-MLLT11)	t(1;19)(q23;p13)(TCF3-PBX1)
t(3;5)(q25;q34)(NPM1-MLF1)	t(3;21)(q26;q22)(RUNX1-MDS1/EV11)
t(4;11)(q21;q23)(MLL-AFF1)	t(5;12)(q33;p13)(ETV6-PDGFRB)
t(5;17)(q35;q21)(NPM1-RARA)	t(6;9)(p23;q34)(DEK-NUP214)
t(6;11)(q27;q23)(MLL-MLLT4)	t(8;21)(q22;q22)(RUNX1-RUNX1T1)
t(9;9)(q34;q34)(SEP-NUT214)	t(9;11)(p22;q23)(MLL-MLLT3)
t(9;12)(q34;p13)(ETV6-ABL1)	t(9;12)(q34;q11)(BCR-ABL1)
t(10;11)(p12;q23)(MLL-MLLT10)	t(11;17)(q23;q21)(MLL-MLLT6)
t(11;17)(q23;q21)(ZBTB16-RARA)	t(11;19)(q23;p13.1)(MLL-ELL)
t(11;19)(q23;p13.3)(MLL-MLLT1)	t(12;21)(p13;q22)(ETV6-RUNX)
t(12;22)(p13;q11)(ETV6-MN1)	t(15;17)(q24;q21)(PML-RARA)
inv(16)(p13;q22)(CBFB-MYH1)	t(16;21)(p11;q22)(FUS-ERG)
t(17;19)(q22;p13)(TCF3-HLF3)	t(;11)(q13;q23)(MLL-FOX04)

step, the slices were dewaxed with phosphate-buffered saline (PBS), baked, and preserved under 4ºC refrigeration until reading of the GSK-3β immunohistochemical staining. GSK-3β immunohistochemical staining was performed in accordance with the instructions of the manufacturer provided by Calbiochem, using biotin-streptavidin and peroxidase for the immunohistochemical technique. PBS was employed to replace the antibody and as a negative control. Images were captured using a digital camera (LABOMED Microscope with an iVU 3100 camera). We used the PIXEL PRO 2.8 color image analysis system to calculate mean optical density (MOD).

Statistical Analysis

For normally distributed variables, we used the Student t-test, and for non-normally distributed variables, we utilized the Mann-Whitney U test. Linear regression analysis, with the predictive variables "deceased" or "alive" and receiver operating characteristics (ROC) curves reporting area under the curve (AUC) and confidence interval (CI) were also employed. Multivariate analysis was performed in order to isolate the GSK-3β and NFKB effect on clinical outcomes. Event free survival (EFS), leukemia-free survival (LFS) and overall survival (OS) were calculated for the examined patients. Any p-value ≤ 0.05 was considered significant. All analyses were performed using SPSS v. 20 statistical software (IBM, Armonk, North Castle, USA).

Ethics

The present study was approved by the HPN Ethics Committee (Code: EI/136/2014) and was conducted in accordance with the Helsinki Declaration (Fortaleza, Brazil). Written informed consent was obtained from parents or patients, and informed consent was obtained from the children.

Results

General Characteristics

Thirty patients were included in this study, with a mean age of 7.1 years (range, 2–13 years), 13 with habitual risk and 17 with high risk. Three patients (one of habitual risk and two of high risk) were referred to another medical hospital. During the first 30 days after treatment with chemotherapy, the EFS for treatment failure (death or relapse) was 86.66%, LFS was 76.66% and OS was 90%. The single relapse case was still alive after the

follow-up period of the study. Three deaths were recorded within the first week after treatment. Table 2 shows the general data of the groups and in Table 3 the same information is disaggregated by the type of leukemia. The majority of patients were male (21), with a female-to-male ratio of 0.3 : 1. Fever, pallor, manifestations of bleeding, hepatosplenomegaly, and lymphadenopathy were common clinical manifestations. No patient had a central nervous system (CNS) disease.

Immunophenotype

Using the morphological classification, 26 patients had L1 and the remaining 4 had L2 ALL type. In 23 patients (76.6%), chromosomal rearrangement analysis revealed the absence of the fusion gene. ETV-RUNX1 was the most common fusion gene abnormality (3 patients), followed by TCF3-PBX1 (2 patients), STL1-TAL1 (1 patient), and BCR-ABL1 (1 patient).

We obtained the results of 28 different chromosomal rearrangements that were related with the development of ALL, without finding significant differences in NFKB expression levels. When analyzing a correlation between clinical features and molecular cytogenetics, it was found that the STL1-TAL1 fusion gene exhibited the lowest hemoglobin level, with a base value of 11.3 g/dL, while the highest level was observed in ALL with the BCR-ABL1 fusion gene, with a mean value of 12.7 g/dL. The TCF3-PBX1 fusion gene demonstrated the lowest white blood cell count, with a base value of $6.55/mm^3$, and the highest count was found in ALL with the STL1-TAL1 fusion gene, with a mean value of $455/mm^3$. The STL1-TAL1 fusion gene showed the lowest platelet count, with a base value of $29/mm^3$, while the highest count was found in ALL with the BCR-ABL1 fusion gene, with a mean value of $370/mm^3$.

Gene Expression

On analyzing the CT values of NFKB expression, the differences between the two groups of risk were not statistically significant. Nonetheless, the mean NFKB expression level showed a trend to be higher in the high-risk than in the habitual-risk group (Table 2).

To analyze whether NFKB demonstrated a relationship with the ALL type, we performed additional analyses according to the immunophenotype pattern, finding statistically significant differences between Pre B-ALL and other ALL types. Interestingly, there were no significant differences either in anthropometric measurements or in hematologic parameters (Table 3).

Table 2. General data of the studied groups*

Characteristic/Variable	Risk	
	habitual 13 (100%)	high 17 (100%)

Sex
  male
  female
	12 (92.30%)
	1 (7.70%)
		9 (52.95%)
		8 (47.05%)

Cytogenetic risk
  present
  absent
	4 (30.77%)
	9 (69.23%)
		3 (17.65%)
		14 (82.35%)

Clinical outcome
  CR
  NCR
	11 (84.61%)
	2 (15.39%)
		11 (64.70%)
		6 (35.30%)

NFKB (RU)		
Age (years)	9 (2–13)	6 (3–13)
Weight (kg)	22.5 (12.30–71.20)	20.00 (13.28–77.00)
Height (cm)	125.00 (80.00–164.00)	126.5 (98.00–176.00)
BMI (kg/m^2)	17.11 (14.10–26.47)	15.61 (13.28–26.22)
Body surface (m^2)	0.90 (0.55–1.81)	0.77 (0.48–1.89)
Waist circumference (cm)	55 (47–63)	57.00 (50.00–104.00)
WBC (c/mm^3)	5.10 (1.70–44.0)	2.70 (1.30–455.00)
Hemoglobin (g/dL)	11.90 (6.20–15.80)	10.90 (5.60–17.60)
Hematocrit (%)	30.15 (26.70–43.70)	31.80 (15.20–53.40)
RBC (c/mm^3)	3.76 (1.88–5.12)	3.73 (1.85–5.78)
Lymphocytes (c/mm^3)	2.88 (0.88–8.20)	1.78 (0.00–6.61)
Monocytes (c/mm^3)	0.15 (0.00–0.70)	0.10 (0.00–161.00)
Eosinophils (c/mm^3)	0.00 (0.00–0.14)	0.00 (0.00–0.06)
Granulocytes (c/mm^3)	0.90 (0.00–7.00)	0.22 (0.00–6.80)
Blasts (%)	0.00 (0.00–97.00)	0.00 (0.00–100.00)
Platelets (c/mm^3)	180 (4–373)	29 (2–407)

* – results expressed in mean and range, except "sex", "cytogenetic risk" and "clinical outcome" which are expressed in frequency and percentage; BMI – body mass index; c – cells; CR – clinical remission; NCR – non-clinical remission; NFKB – nuclear factor kappa B; RBC – red blood cells; RU – relative units; WBC – white blood cells.

Immunohistochemistry

The immunohistochemistry of GSK-3β revealed 8 patients who were positive, 8 patients who were weak-positive, and 15 patients who were negative. Table 4 illustrates the distribution of immunohistochemistry results of the bone marrow samples by risk classification.

Through the Pearson test, there was a positive correlation between high-risk patients and the GSK-3β expression (R = 0.741; p = 0.010). Interestingly, by doing an intragroup correlation with NFKB expression, those patients with a positive or weak-positive result in GSK-3β showed a positive correlation with NFKB expression (R = 0.910; p ≤ 0.001).

When grouping patients under two conditions, that is, a) failure (deceased or relapsed) and b) alive with typical treatment, there were 5 variables with a statistically significant difference: body surface (p = 0.034); urea (p = 0.036); blood urea nitrogen (BUN) (p = 0.044); lactate dehydrogenase (LDH) (p = 0.027), and total bilirubin (TB) (p = 0.006).

By means of the linear regression analysis, predictive variables for "deceased" or "alive" comprised uric acid (mg/dL), hemoglobin (g/dL), and GSK-3β

Table 3. General data of the patients by type of leukemia

Characteristic/ Variable	Pre B-late 11 (36.66%)	Pre B 6 (20.00%)	Pre B common 2 (6.66%)	T 3 (10.00%)	B 8 (26.66%)	P-value
Sex Male (n) Female (n)	5 6	6 0	2 0	3 0	5 3	
Age (years)	10 (2–13)	5 (2–12)	12 (11–13)	9 (6–11)	3 (2–7)	0.052
Weight (kg)	27.00 (12.50–71.20)	15.57 (12.60–77.00)	40.25 (36.00–44.50)	22.5 (19.40–26.00)	13.28 (12.30–20.00)	0.096
Height (cm)	139.00 (92.00–165.00)	94.00 (80.00–176.00)	143.00 (136.00–150.00)	125.00 (118.00–134.00)	99.00 (91.00–119.00)	0.038
BMI (kg/m^2)	16.37 (13.50–26.47)	19.6 (15.84–24.86)	19.62 (19.46–19.78)	14.40 (13.93–14.48)	14.86 (13.28–17.35)	0.617
Body surface (m^2)	0.95 (0.48–1.81)	0.61 (0.58–1.89)	1.29 (1.20–1.38)	0.86 (0.77–0.96)	0.70 (0.55–0.79)	0.204
Waist circumference (cm)	53.0 (50.00–87.00)	55.0 (51.00–104.00)	64.00 (52.00–76.00)	57.5 (57.00–60.00)	52.50 (47.00–57.00)	0.304
WBC (c/mm^3)	3.60 (1.30–44.00)	6.45 (1.70–11.70)	4.95 (2.30–7.60)	3.90 (2.20–445.00)	2.30 (2.00–10.80)	0.026
Hematocrit (%)	32.50 (17.20–45.80)	25.45 (20.30–31.60)	25.75 (15.20–36.30)	31.50 (26.20–39.20)	40.10 (31.80–53.40)	0.409
RBC (c/mm^3)	3.78 (1.88–5.04)	3.23 (2.31–3.89)	3.02 (1.85–4.19)	3.43 (2.94–4.45)	4.60 (3.73–5.78)	0.271
Hemoglobin (g/dL)	12.00 (6.20–14.70)	9.15 (6.90–11.90)	9.15 (5.60–12.70)	11.30 (8.70–13.50)	13.50 (10.90–17.60)	0.189
Platelets (c/mm^3)	35.00 (4.00–359.00)	96.50 (2.00–373.00)	263.50 (157.00–370.00)	153.00 (29.00–216.00)	66.00 (7.00–407.00)	0.497
NFKB (RU)	0.40 (0.00–29.10)	2.25 (0.20–113.70)	3.20 (0.10–6.30)	0.10 (0.10–21.10)	0.50 (0.00–2.80)	0.049[*]

BMI – body mass index; c – cells; NFKB – nuclear factor kappa B; RBC – red blood cells; RU – relative units; WBC – white blood cells; * significance ≤ 0.05.

(OD relative units). These results were constant on analyzing only males and when the groups were "failure" and "alive with typical treatment".

In the ROC analysis, when the options were "deceased" vs. "alive", showed TB (AUC: 932, CI: 0.794–1.070) to be the only significant prognosis variable (p = 0.013); while using the same approach with the options "failure" vs. "alive with typical treatment", yielded GSK-3β (AUC: 962, CI: 0.858–1.065) as the only significant prognosis variable (p = 0.042).

Finally, GSK-3β (OD relative units) had a significant value (p = 0.037) as a survival prognosis factor when introducing the "risk classification" and the presence of "cytogenetic alterations" as factors in the multivariate lineal regression model.

Discussion

ALL is a disease in which micro-environmental interactions and abnormalities in the genome and the epigenome accumulate, enabling cells to avoid the control mechanisms of the cell cycle. This affects the effectiveness of the repair systems as a result of exposure to harmful endo- and exogenous damaging agents [8, 9].

We did not find statistically significant differences in hematic biometry by risk stage, outcome or ALL type, this suggesting that implementation of molecular biomarkers is one of several requirements for the prognosis of childhood ALL. *ETV-RUNX1* and *TCF3-PBX1* fusion genes were found in a higher proportion of cases compared to that reported in literature [10].

Table 4. GSK-3 β and surface markers*

Marker	Detection	RISK	
		Habitual 13 (100%)	High 17 (100%)
GSK-3β	positive	0 (0.00%)	8 (47.06%)
	weak positive	5 (38.47%)	3 (17.64%)
	negative	9 (69.23%)	6 (35.30%)
CD3	positive	0 (0.00%)	2 (11.76%)
	negative	13 (100.00%)	15 (88.24%)
CD5	positive	0 (0.00%)	1 (5.88%)
	negative	13 (100.00%)	16 (94.12%)
CD10	positive	11 (84.62%)	12 (70.59%)
	negative	2 (15.38%)	5 (29.41%)
CD13	positive	1 (7.69%)	0 (0.00%)
	negative	12 (92.31%)	13 (100.00%)
CD19	positive	12 (92.31%)	13 (100.00%)
	negative	1 (7.69%)	4 (23.53%)
CD20	positive	2 (15.38%)	3 (17.65%)
	negative	11 (84.62%)	14 (82.35%)
CD22	positive	8 (61.54%)	10 (58.82%)
	negative	5 (38.46%)	7 (41.18%)
CD34	positive	7 (53.85%)	6 (35.29%)
	negative	6 (46.15%)	11 (64.71%)
CD45	positive	12 (92.31%)	15 (88.24%)
	negative	1 (7.69%)	2 (11.76%)
CD79a	positive	12 (92.31%)	13 (76.47%)
	negative	1 (7.69%)	4 (23.53%)
CD117	positive	2 (15.38%)	4 (23.53%)
	negative	11 (84.62%)	13 (76.47%)
HLADR	positive	12 (92.31%)	13 (76.47%)
	negative	1 (7.69%)	4 (23.53%)
MPO	positive	0 (0.00%)	1 (5.88%)
	negative	13 (100.00%)	16 (94.12%)
TdT	positive	7 (53.85%)	7 (41.18%)
	negative	5 (38.46%)	10 (58.82%)

* – results expressed in frequency and percentage.

Immunophenotype and chromosomal alterations comprise some of the several outcome tools in patients with ALL. However, we did not find significant differences between the two clinical-risk groups (high and habitual) related to the expression of cell surface markers.

In human tumorigenesis, *NFKB* is an important factor in cancer cell survival [11]. In fact, alterations to the *NFKB* pathway are particularly recognized in ALL and other leukemias, for example, its regulation should facilitate the apoptosis of leukemic cells [12].

Recently, GSK-3β has been associated with the regulation of *NFKB* activity. For first instance, GSK-3β inactivation decreases the activation pathway of *NFKB*, generates gene suppression, and stimulates apoptosis *in vitro* [13]. According to Hu in 2011, GSK-3β inhibitors significantly decreased *NFKB* expression, suggesting that it is a new, interesting target in ALL treatment, these results supporting the *NFKB*/GSK-3β relationship [14, 15]. In our analysis, immunohistochemistry to GSK-3β demonstrated 8 patients who were positive, 8 patients who were weak-positive, and 15 patients who were negative, these results showing a positive correlation with *NFKB* expression levels. However, *NFKB* expression and its role in the development of ALL may be a result of interactions with GSK-3β, a perspective to analyze.

To date, there has been scarce information explaining the role of GSK-3β in relation to survival and proliferation pathways [16, 17]. In this line of research, our previously mentioned results indicate that increased expression of GSK-3β may be directly associated with regulation of the *NFKB* pathway. In addition, the function of *NFKB* and GSK-3β in leukemic cells indicates that this molecular pathway might be a potent therapeutic target [18, 19], and that prognoses should obtain better results utilizing them.

In pediatric ALL cells, the GSK-3β/*NFKB* relationship has already been studied [20–23]; however, while the results suggest an influence of GSK-3β on the transcriptional activity of *NFKB p65*, there were no changes in its expression in this initial approach. On the other hand, the use of selective GSK-3β inhibitors is a target in the treatment of pediatric ALL. Furthermore, *NFKB* expression levels and GSK-3β should be associated with the clinical risk of the pediatric patients.

A limitation of our study is the lack of comparison among different chemotherapy schemes, but the number of patients needed to do so, implies a time beyond the perspective of our main objective, which is to evaluate new options of prognosis markers in the pediatric population attended in Mexico.

In conclusion, the current study revealed an interaction between the *NFKB* expression levels and GSK-3β signaling pathways in patients with ALL. Thus, the GSK-3β expression pattern needs to be studied as a prognosis factor, without ruling out other signaling pathways such as PI3K/PTEN/Akt/mTOR and Ras/Raf/MEK/ERK and the mammalian target of rapamycin (mTOR).

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