UNIVERSIDAD AUTÓNOMA DE SAN LUIS POTOSÍ

FACULTAD DE CIENCIAS QUÍMICAS

PROGRAMA DE POSGRADO
EN CIENCIAS FARMACOBIOLÓGICAS

TÍTULO DEL TRABAJO

"EVALUACIÓN DEL ESTADO EPIGENÉTICO DE LAS N-ACETILTRANSFERASAS NAT1 Y NAT2 EN PACIENTES PEDIÁTRICOS CON LEUCEMIA LINFOBLÁSTICA AGUDA"

TESIS PARA OBTENER EL GRADO DE
DOCTORADO EN CIENCIAS FARMACOBIOLÓGICAS

PRESENTA
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San Luis Potosí, S.L.P. OCTUBRE 2020

Proyecto realizado en:

Departamento Hemato–Oncología Pediátrica del Hospital Central “Dr. Ignacio Morones Prieto”.

Laboratorio de Medicina Molecular y Traslacional del Centro de Investigación en Ciencias de la Salud y Biomedicina, de la Universidad Autónoma de San Luis Potosí.

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***No te avergüences por tus fracasos; aprende de ellos y comienza de nuevo*.**

Richard Branson

"Todo tiene su tiempo, y todo lo que se quiere debajo del cielo tiene su hora. Tiempo de nacer, y tiempo de morir; tiempo de plantar, y tiempo de arrancar lo plantado; tiempo de matar, y tiempo de curar; tiempo de destruir, y tiempo de edificar; tiempo de llorar, y tiempo de reír; tiempo de endechar, y tiempo de bailar; tiempo de esparcir piedras, y tiempo de juntar piedras; tiempo de abrazar, y tiempo de abstenerse de abrazar; tiempo de buscar, y tiempo de perder; tiempo de guardar, y tiempo de desechar; tiempo de romper, y tiempo de coser; tiempo de callar, y tiempo de hablar; tiempo de amar, y tiempo de aborrecer; tiempo de guerra, y tiempo de paz".

Eclesiastés 3

Aprendí que un tropezón no es una caída…
Que todo en la vida vuelve…
Que no hay mal que por bien no venga…
Con voluntad y esfuerzo, todo resulta más fácil…
Que lo más valioso en el mundo es, la familia y los amigos de verdad…
Que no se llora a quien no te valora…
Que, por más tropezón, caída u obstáculo, o barrara que se interponga en el camino, el objetivo es levantar la cabeza y SEGUIR ADELANTE…

Autor desconocido

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RESUMEN

La leucemia linfoblástica aguda (LLA) es la neoplasia más frecuente en población infantil y actualmente su etiología continua en investigación. Se ha propuesto que la presencia de polimorfismos en los genes de las N-Acetiltransferasas (NATs) NAT1 y NAT2 son un factor causal; además, no se conocen los niveles de expresión y actividad de las NATs en células inmunes de pacientes con LLA. En este trabajo, se aislaron células mononucleares de sangre venosa periférica de un grupo control donde se incluyó niños sanos (n= 19) y un grupo de pacientes con LLA (n=20). Se determinó la expresión de ARNm por PCR en tiempo real, el porcentaje de células positivas para NAT1 y NAT2 por citometría de flujo y la actividad enzimática mediante un cultivo celular con sustrato específico por HPLC. Se observaron niveles bajos de expresión de NAT1 a nivel de ARNm ($p=0.001$) y de proteína ($p=0.0003$) en los pacientes con LLA, así como en la actividad enzimática ($p=0.0047$) cuando se comparó con el grupo control. En linfocitos T $CD3^+$ se detectó una baja expresión de NAT1, principalmente en aquellos pacientes que presentaron recaída a la neoplasia. Mediante ensayos de CHIP se encontró una menor acetilación de histonas en el promotor de NAT1, lo que podría explicar la baja expresión del ARNm. Cuando se analizó NAT2, se detectó una menor expresión a nivel de proteína solo en células $CD3^+$ (linfocitos T) ($p=0.04$). En conclusión, la enzima metabolizadora de fármacos NAT1 podría ser considerada como un factor de mal pronóstico en la LLA y podría influir en el desarrollo de la neoplasia.

Palabras clave: Leucemia, NAT1, NAT2, expresión, actividad enzimática.

ABSTRACT

Acute lymphoblastic leukemia (ALL) is the most common neoplasm in children and whose etiology continues to be studied. N-Acetyltransferases are drug-metabolizing enzymes, and NAT1 participates in carcinogenesis. The expression and activity levels of NAT1 or NAT2 in ALL are unknown. Therefore, expression at the level of mRNA and protein was evaluated, as well as the enzymatic activity of NAT1 and NAT2 in pediatric patients with ALL and apparently healthy children. Peripheral venous blood mononuclear cells (PBMC) were isolated in a control group (n=19) and with ALL (n=20, 7 presented relapse). mRNA was evaluated by real-time PCR and the percentage of cells positive for NAT1 and NAT2 by flow cytometry; enzymatic activity was determined by HPLC from a cell culture with the specific substrate. Low levels of NAT1 mRNA expression (p=0.001) and protein (p=0.0003), as well as enzymatic activity (p=0.0047) were observed in PBMC from children with ALL compared to the control group. Through the t-SNE analysis, NAT1 was found to have lower expression in $CD3^+$ lymphocytes of patients who relapsed with respect to the first diagnosis. NAT1 is present only in $CD19^+$ lymphocytes in the control group, but not in ALL patients with relapse. The CHIP assays showed less histone acetylation in the NAT1 promoter, which could explain the low expression of mRNA. In contrast, NAT2 showed similar levels of mRNA expression (p=0.51) but higher level of protein expression in CD3+ lymphocytes (p=0.04) of patients with ALL than in the control group, however, the enzyme activity was similar between the groups (p=0.2). The results indicate that NAT1 could be used as a poor prognostic factor and that it could influence the development of the neoplasia.

ÍNDICE

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1. Introducción

La leucemia linfoblástica aguda (LLA) es la neoplasia maligna que se diagnostica con mayor frecuencia en pacientes pediátricos. Se presenta con mayor número incidencia entre los 3 y 5 años de edad y representa del 25 al 30% de todos los tipos de neoplasias malignas infantiles. México es el segundo país de América Latina con mayor número de casos de prevalencia e incidencia de LLA (1). Se desconocen las causas de la alta incidencia, sin embargo, se ha propuesto que las enzimas que participan en el proceso de detoxificación podrían influir en el desarrollo de la enfermedad. Dentro de las enzimas metabolizadoras de xenobióticos, se encuentran las Arilaminas N-Acetiltransferasas (NATs), NAT1 y NAT2. Los genes que codifican para estas enzimas son altamente polimórficos, lo que cual genera 3 fenotipos de acetilación: lento, rápido e intermedio. Los acetiladores lentos de NAT2 son menos eficientes en el metabolismo de aminas aromáticas, esto ocasiona que dichas moléculas permanezcan más tiempo en el organismo, lo cual podría favorecer la generación de aductos en el ADN y causando mutaciones. Por otra parte, los acetiladores rápidos NAT1 convierten, en una mayor proporción, los compuestos xenobióticos (compuestos tóxicos, específicamente) a productos altamente reactivos, lo que podría ocasionar que exista una alta probabilidad de presentarse mutaciones debido a la formación de aductos de ADN (2).Estudios recientes llevados a cabo en una línea celular derivada de adenocarcinoma de colon (HT-29) asocian a NAT1 con el proceso de carcinogénesis debido a su participación en la regulación de la expresión de p53 (3). Además, utilizando una línea celular de cáncer de mama (MDA-MB-231), se demostró que la sobreexpresión de NAT1 tiene como resultado la disminución en la cantidad de Acetil coenzima A, acumulándose Coenzima A, lo cual podría inducir apoptosis por las altas concentraciones de este compuesto o bien, alterar otros mecanismos celulares debido a la participación de la acetil coenzima A en diversos procesos fisiológicos (4).

Nuestro grupo de investigación determinó que existen asociaciones con los haplotipos *NAT1*4* (OR=1.92), *NAT2*6B* (OR=3.3) (590G>A), *NAT2*6J* (OR=3.25) (282C>T,590G>A, 857G>A) y *NAT2*7A* (OR=2.45) (857 G>A) en pacientes pediátricos con LLA mestizos mexicanos (5). Sin embargo, se desconocen los niveles de expresión y regulación a nivel de proteína, ARNm y de actividad enzimática en esta neoplasia, los cuales podrían verse alterados en pacientes con LLA y por lo tanto, estos datos contribuirían con información clave que podría elucidar la etiología de esta neoplasia.

2. Objetivos

2.1 Objetivo General

Evaluar el estado de metilación del ADN y el de acetilación y metilación de histonas en los promotores de los genes *NAT1* y *NAT2* y asociarlos con los niveles de expresión y función del gen y de la enzima en células mononucleares de sangre venosa periférica (PBMC) de sujetos control y pacientes pediátricos con leucemia linfoblástica aguda.

2.2 Objetivos Específicos

- Analizar el patrón de metilación de ADN en los promotores de los genes NAT's en PBMC de sujetos control y pacientes con leucemia linfoblástica aguda mediante la técnica de PCR en tiempo real y secuenciación por bisulfito.
- Evaluar el estado de acetilación y metilación de histonas en los promotores de los genes *NATs* en PBMC de sujetos control y pacientes con leucemia linfoblástica aguda mediante la técnica de inmunoprecipitación de la cromatina (CHIP).
- Determinar la expresión de NATs a nivel ARNm y proteína en PBMC por PCR en tiempo real y citometría de flujo en sujetos control y pacientes con leucemia linfoblástica aguda.
- Evaluar la actividad de las enzimas NATs por cultivo celular con sustratos específicos en PBMC de sujetos control y pacientes con leucemia linfoblástica aguda mediante la técnica de HPLC.
- Realizar el análisis de asociación de patrón de metilación de ADN y el de acetilación y metilación de histonas con los niveles de expresión y actividad enzimática de las NATs.

3. Material y métodos

El presente trabajo fue un estudio observacional, transversal, piloto, analítico y prospectivo. El protocolo se aprobó por los Comités de Investigación y de Ética en investigación del Hospital Central “Dr. Ignacio Morones Prieto” (HCDIMP) (Registro 25-17). Participaron pacientes del Área de Oncopediatría y Servicio de Pediatría, atendidos en el HCDIMP, que cumplieron con los criterios de inclusión de este estudio. Se obtuvo el consentimiento informado del padre o tutor y el asentimiento si el menor era mayor de 12 años de edad hasta los 17 años, 11 meses y 29 días.

3.1 Toma de muestras biológicas y aislamiento de células Mononucleares

Para ambos grupos se tomó, mediante una venopunción al momento de estudios de rutina de los participantes, una muestra única de 4 mL de sangre periférica anticoagulada con EDTA. Se aislaron las PBMC por gradiente de densidad mediante Ficoll-Hypaque, las cuales se emplearon para los distintos ensayos.

3.2 Ensayo de inmuno precipitación de la cromatina (CHIP)

Las PBMC se fijaron con formaldehído al 1% y posteriormente se sonicaron en el equipo EpiSonic™ 2000 Sonication System, para obtener fragmentos de ADN de aproximadamente 300-700 pb. Los complejos de proteína-ADN se inmunoprecipitaron con perlas magnéticas (Dynabeads Protein G, Thermofisher) y los anticuerpos anti-histona H3 (Abcam® ab1791), anti-histona H3 trimetil K27) (Abcam® ab6002) y anti-acetilhistona H3 (K14) (Merck Millipore®). El ADN inmunoprecipitado se purificó y se utilizó para realizar los ensayos de PCR.

3.3 Expresión del ARNm

El ARN se obtuvo de las PBMC utilizando TRIzol® (Thermo Fisher Scientific®). La síntesis de ADN complementario se realizó con el ensayo comercial High Capacity cDNA Reverse Transcription (Applied Biosystems®). La expresión relativa del ARNm se midió mediante mediante PCR en tiempo real utilizando SYBR Green qPCR Master Mixes (Thermo Fisher Scientific) y empleando β-actina como gen endógeno. El nivel de expresión se calculó mediante el método $2^{-\Delta\Delta Ct}$.

3.4 Determinación de la expresión de proteína

La cantidad de proteína de cada enzima en las PBMC se determinó mediante citometría de flujo utilizando el equipo FACSCanto II instrument (Beckton-Dickinson). Se emplearon anticuerpos específicos de superficie específicos para CD3-PE (Clona HIT3a) y CD19-FITC (Clona HIB19) (eBiosciences®). Para determinar la expresión de las proteínas NAT1 y NAT2, las células se fijaron y se permeabilizaron utilizando el ensayo comercial Fixation/Permeabilization (eBiosciences®) y se utilizaron los anticuerpos intracelulares: NAT1-APC y NAT2-APC (ab109114 y ab88443 Abcam®). Los resultados obtenidos se analizaron en el programa FlowJo Versión 10.4.

3.5 Determinación de la actividad enzimática por HPLC

Las PBMC se cultivaron en medio RPMI® en placas de ELISA de 48 pozos por 24 horas con los respectivos sustratos. Para NAT1 se empleó como sustrato el ácido para-amino benzoico (Sigma Aldrich®) y para NAT2 se empleó como sustrato Isoniazida (Sigma Aldrich®). La fase móvil consistió en 80% de ácido acético 50 mM y 20% de acetonitrilo, a una λ de 270 nm para NAT1, y 97% de Heptanosulfonato de sodio 20 mM con Buffer de fosfatos 2.5 mM y 3% acetonitrilo a una λ de 266 nm para NAT2. 20 µL de muestra se analizaron mediante la técnica de HPLC. El equipo que se utilizó fue el Cromatógrafo de Líquidos de Alta Resolución (Waters System, Milford, MA, USA) con detector UV-Vis Waters modelo 2487. La columna y precolumna empleadas fueron de la marca Waters X-terra RP18.

3.6 Análisis estadístico

Cuando los datos fueron paramétricos, se compararon las medias mediante la prueba de T-Student; si los datos resultaron no paramétricos, se compararon las medianas con U de Mann-Whitne,. El análisis estadístico se realizó con el software GraphPad Prism V 7.00 (Graphpad Software Inc., CA, USA).

4. Resultados y discusión

En este estudio participaron 19 niños control (10 hombres y 9 mujeres) entre los 4 y 15 años; y 20 niños con LLA (12 hombres y 8 mujeres) entre los 3 y 15 años. Del grupo de niños con LLA, 19 se clasificaron como pacientes de alto riesgo y 1 como paciente de riesgo habitual. 7 de los 20, presentaron recaída. Todos los pacientes fueron diagnosticados como LLA del tipo B por el oncólogo pediatra. La mayoría de los pacientes se encontraban en fase de mantenimiento (n=17), uno en consolidación y dos en reinducción al tratamiento. La clasificación EGIL mostró lo siguiente; EGIL B1 (n=2), EGIL B2 (n=9) y EGIL B3 (n=9).

Nuestros resultados muestran que existe una disminución en la expresión del ARNm ($p=0.001$) y de la proteína NAT1 en términos de valores absolutos ($p=0.0003$) en pacientes con LLA respecto al grupo control.

Los ensayos de evaluación de la actividad enzimática de NAT1 demostraron que existe una expresión baja de la misma en pacientes con LLA ($p=0.0047$), incluso, algunos sujetos presentaron actividad nula. Estos resultados coinciden con reportes en líneas celulares de leucemia (THP-1, Jurkat o CEM), cáncer de hígado (HepG2) y colon (HT-29); en contraste, en líneas celulares de cáncer de mama (T-47D, ZR-751) y próstata (LNCaP, 22RV1) se ha reportado que la actividad de NAT1 se encuentra elevada. Se desconocen las causas de este comportamiento opuesto, sin embargo, las modificaciones postraduccionales y/o los mecanismos epigenéticos, los cuales varían entre distintos tipos de cáncer podrían estar jugando un papel importante.

La baja expresión y actividad de NAT1 podría ser un factor influyente en el proceso de carcinogénesis, debido a que podrían existir grandes concentraciones intracelulares de Acetil coenzima A, lo cual favorece la proliferación celular. Por lo tanto, es necesario evaluar las concentraciones de este cofactor en células leucémicas.

El análisis de expresión de NAT1 en linfocitos T $CD3^{+}$, determinó que aquellos que mostraban baja expresión de NAT1, se caracterizaban por presentar recaída a la neoplasia, lo que nos sugiere que una disminución en la expresión de NAT1 en esta subpoblación celular podría ser utilizado como un factor de mal pronóstico. Los resultados del ensayo de CHIP, mostraron en un paciente con LLA disminución de los

niveles de acetilación en histona H3 lisina 14 (H3K14ac) en el promotor de NAT1 respecto al sujeto control, lo cual podría explicar la menor expresión del mensajero.

No se observaron diferencias estadísticas entre los grupos de estudio en la expresión del ARNm, proteína y actividad enzimática cuando se analizó NAT2. Sin embargo, en el ensayó de CHIP se detectaron menores de niveles de acetilación en H3K14ac y mayores niveles de metilación en histona 3 lisina 27 (H3K27me3) en un paciente con LLA respecto al sujeto control, aunque no se reflejara el efecto en la supresión del mensajero. Sin embargo, la cromatina analizada fue proveniente de PBMC, con base a los resultados de citometría de flujo, es necesario realizar el estudio de CHIP por subpoblación celular (T y B) específica. En contraste, los niveles de células dobles positivas $CD3^+/NAT2^+$ ($p=0.04$) fueron mayores en pacientes con LLA. El resultado obtenido fue opuesto al esperado, ya que NAT2 es afín a la reacción de N-acetilación, una reacción que provee un efecto protector al desarrollo de cáncer, ya que se favorece la detoxificación de los xenobióticos.

5. Conclusiones

El presente estudio describe por primera vez la expresión y actividad enzimática de NAT1 y NAT2 en PBMC de pacientes con LLA. Nuestros datos indican que la expresión de NAT1 en linfocitos $CD3^+$ podría ser un factor de mal pronóstico en los pacientes con LLA y que las alteraciones encontradas en esta enzima podrían influir en el desarrollo de cáncer, ya que se ha descrito a NAT1 con una posible influencia en la regulación del ciclo celular. Para NAT2 no se detectaron alteraciones en los distintos ensayos realizados en las PBMC de los pacientes con LLA.

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ARTÍCULOS DE INVESTIGACIÓN

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Abstract:	Background: Acute lymphoblastic leukemia (ALL) is the most common neoplasm in children and whose etiology continues to be studied. N-Acetyltransferases are drug-metabolizing enzymes, and NAT1 participates in carcinogenesis. The expression and activity levels of NAT1 or NAT2 in the carcinogenesis process of ALL are unknown. Objective: We aimed to evaluate the expression at the mRNA and protein levels, as well as the enzymatic activity of NAT1 and NAT2 in pediatric patients with ALL and apparently healthy children. Patients and Methods: Peripheral venous blood mononuclear cells (PBMC) were isolated in a control group (n=19) and with ALL (n=20, 7 presented relapse). mRNA was evaluated by real-time PCR and the percentage of cells positive for NAT1 and NAT2 by flow cytometry; enzymatic activity was determined by HPLC from a cell culture with the specific substrate. Results: Low levels of NAT1 mRNA expression (p=0.001) and protein (p=0.0003), as well as enzymatic activity (p=0.0047) were observed in PBMC from children with ALL compared to the control group. The t-SNE analysis demonstrated that NAT1 had lower expression in CD3+ lymphocytes of patients who relapsed with respect to the first diagnosis patients. NAT1 is present only in CD19+ lymphocytes in the control group, but not in ALL patients with relapse. The CHIP assays showed less histone acetylation in the NAT1 promoter, which could explain the low expression of mRNA. In contrast, NAT2 showed similar levels of mRNA expression (p=0.51) but a higher level of protein expression in CD3+ lymphocytes (p=0.04) of patients with ALL than in the control group, however, the enzyme activity was similar between the groups (p=0.2). Conclusions: The results indicate that NAT1 could be used as a poor prognostic factor and that it could influence the development of the neoplasia.
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Alteration of expression levels and enzymatic activity of arylamine N-acetyltransferases 1 and 2 related to clinical relapse in acute lymphoblastic leukemia

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Abstract

Acute lymphoblastic leukemia (ALL) is the most common neoplasm in children and whose etiology continues to be studied. N-Acetyltransferases are drug-metabolizing enzymes, and NAT1 participates in carcinogenesis. The expression and activity levels of NAT1 or NAT2 in ALL are unknown. Peripheral venous blood mononuclear cells (PBMC) were isolated in a control group (n=19) and with ALL (n=20, 7 presented relapse). mRNA was evaluated by real-time PCR and the percentage of cells positive for NAT1 and NAT2 by flow cytometry; enzymatic activity was determined by HPLC from a cell culture with the specific substrate. Low levels of NAT1 mRNA expression (p=0.001) and protein (p=0.0003), as well as enzymatic activity (p=0.0047) were observed in PBMC from children with ALL compared to the control group. Through the t-SNE analysis, NAT1 was found to have lower expression in $CD3^+$ lymphocytes of patients who relapsed with respect to the first diagnosis. NAT1 is present only in $CD19^+$ lymphocytes in the control group, but not in ALL patients with relapse. The CHIP assays showed less histone acetylation in the NAT1 promoter, which could explain the low expression of mRNA. In contrast, NAT2 showed similar levels of mRNA expression (p=0.51) but higher level of protein expression in CD3+ lymphocytes (p=0.04) of patients with ALL than in the control group, however, the enzyme activity was similar between the groups (p=0.2). The results indicate that NAT1 could be used as a poor prognostic factor and that it could influence the development of the neoplasia.

1. Introduction

Acute lymphoblastic leukemia (ALL) is the most common malignancy diagnosed in pediatric patients and represents 25–30% of all types of childhood cancer (1-3). Within Latin America, Mexico has the second highest number of cases and prevalence and incidence of this disease (4). It is estimated that 650 to 780 cases of ALL are detected in our country per year, with ALL representing the malignancy with the highest cause of mortality in children from 5 to 14 years old (5, 6). The possible causes of the high incidence of ALL and its etiology is still under study, however, genetic factors (caused by a mixture of Indigenous and European heritage miscegenation) and those related to lifestyle contribute greatly (1, 4).

Exposure to chemical agents (pesticides, paints or chemicals intended for household chores), annealing of meat foods, smoke pollutants, and parental smoking, together with immaturities in physiological development in children, are important contributors in the development of ALL (7, 8). The phase I enzymatic reactions of such xenobiotics catalyzed by the cytochrome P450 complex can activate distinct compounds—polycyclic aromatic hydrocarbons and aromatic and heterocyclic amines—and induce the generation of carcinogenic compounds. The accumulation of active carcinogenic metabolites can cause an increase in the formation of DNA adducts, which can lead to an increase in the risk of developing some types of cancer, including LLA (9, 10). However, the resulting intermediate compounds, that are highly electrophilic and reactive, can be detoxified or transformed to less potent compounds through the action of phase 2 enzymes (3, 11).

The arylamine N-Acetyltransferases (NATs), NAT1 and NAT2, are important phase 2 xenobiotic metabolizing enzymes that participate in detoxification reactions through the addition of an acetyl group from CoA to arylamine compounds. The genes NAT1 and NAT2, which share 87% nucleotide homology, are located on chromosome 8 (NAT1 at 8p21.3-23.1 and NAT2 at 8p21.3-23.1 and 8p22) and are inherited co-dominantly (12, 13). Several Single Nucleotide Polymorphisms (SNPs) have been described in both these genes and their presence related to the likelihood of development of ALL (3, 11,

14, 15). Additionally, NAT1 could be involved in the cell cycle and apoptosis through regulation of p53, a tumor suppressor protein, and, as a consequence, participate in the generation of reactive oxygen species (16). Moreover, elevated levels of NAT1 have been associated with a low amount of acetyl coenzyme A (Acetyl-CoA) which can induce cell apoptosis (17, 18). Furthermore, it has been proposed that NAT1 could become a therapeutic target since it has been described that tamoxifen or cisplatin can inhibit the activity of this enzyme in breast cancer (19).

Previously, our group demonstrated the presence of these enzymes in mononuclear cells and described the significant associations between the development of ALL and the presence of the haplotypes *NAT1*3* (Odds ratio [OR], 2.1), *NAT1*4* (OR, 1.9), *NAT2*6B* (OR, 3.3), *NAT2*6J* (OR, 3.2), *NAT2*7A* (OR, 2.4), and the NAT1 rapid (OR, 6.7) and NAT2 slow phenotypes (OR, 2.9) (14), coinciding with the genetic association studies from other countries (3, 11, 15). Therefore, NAT1 and NAT2 are molecules that could be key in the development and progression of ALL.

To date, the levels of expression of these genes remains unexplored. Moreover, there is a need to determine the activity and function of the xenobiotic metabolizing enzymes in this pediatric neoplasm and establish whether they have any implication in the development of the leukemia. Therefore, the objective of this study was to evaluate the expression of the arylamine N-Acetyltransferases 1 and 2 at the mRNA and protein level, as well as determine their enzymatic activity in peripheral blood mononuclear cells and also in $CD3^+$ or $CD19^+$ lymphocytes obtained from patients with ALL, as compared to a control group.

2. Materials and methods

2.1. Subjects

In the present study, 20 unrelated pediatric patients, aged 3 to 15 years, with confirmed diagnosis of ALL and 19 clinically healthy children (control group) were recruited from the Hospital Central “Dr. Ignacio Morones Prieto” (HCIMP) of San Luis Potosí, Mexico. In general, parents gave written informed consent, however, for patients aged 12 to 17

years, informed assent was obtained directly from the patient. ALL diagnosis was confirmed by flow cytometry analysis and several monoclonal antibodies against CD10, CD19, CD20, CD22, CD34, CD79a, TdT, IgMs, and IgMc were used. All patients were classified as subtype B and received oncological treatment described in the Mexican clinical guidelines, with drugs such as mercaptopurine, methotrexate, and L-asparaginase (20). The protocol was approved by the research committee and the research ethics committee of HCIMP (number 25-17).

Patients included in the study were assigned to a risk group based on predictive factors established in the literature (20-22): 19 out of 20 were at high risk, and only one was assigned to the low risk group. After 2 years of study follow-up, 2 patients died and 7 of the 20 ALL patients had been diagnosed with relapses (patients who returned to have blasts in their monitoring studies according to the monitoring guidelines (20). Among the concomitant diseases in patients with ALL, one patient presented with Down syndrome and another with hyperthyroidism. Body mass index (BMI) was calculated according to age and sex, as established by the world health organization (WHO) (23). It was taken into account that patients with ALL and those of the control group were not related to each other.

2.2. Isolation of peripheral blood mononuclear cells

Blood samples were collected from both groups in 4 mL EDTA Vacutainer tubes (BD VACUTAINER®) for the NATs expression and enzymatic activity analysis. Peripheral blood mononuclear cells (PBMC) from controls and ALL patients were isolated by Ficoll gradient centrifugation. Blood was diluted with an equal volume of phosphate-buffered saline (PBS) pH=7.3, overlaid on layered Ficoll-Histopaque (Sigma, St. Louis, MO, USA) and centrifuged at 2500 rpm (500×g) for 20 min at 25°C. Cell viability was assessed by trypan blue exclusion assay and a minimum of 90% viable cells was required to perform further experiments.

2.3. NAT1 and NAT2 mRNA expression assay

Total RNA from PBMC from each participant was purified using the TRIzol® reagent. The integrity of RNA was assessed by electrophoresis using 1% agarose gel and the quality and yield were evaluated using a Synergy® HT Multi-Mode Microplate Reader (BioTek Instruments, VT, USA). 500 ng for each RNA sample was used to generate cDNA with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, CA, USA), according to the manufacturer's recommendations. 300 ng of cDNA was used to perform Real-Time PCR.

NAT1 and NAT2 mRNA expression was determined using the specific primers: NAT1 Fw 5'-GAATTCAAGCCAGGAAGAAGCA-3'; Rv 5'-TCCAAGTCCAATTTGTTCCTAGA CT-3'; and NAT2 Fw 5'-GATCACTTCCCTTGCAGA CTTT-3' Rv 5'-AGGCTGAATGCAATCCTCTTG-3'. The reactions were performed using CFX 96 Real-Time PCR (Bio-Rad Laboratories, CA, USA) using the iQ™ SYBR® Green Supermix (Bio-Rad Laboratories, CA, USA). The thermocycling conditions were: 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Expression of the NATs genes was normalized against the level of the control endogenous β-actin, with the primers Fw 5'-GTCCACCTTCCAGCAGATGTG-3' and Rv 5'-GCATTTGCGGTGGACGAT-3', using the $2^{-\Delta\Delta Ct}$ method (24, 25).

2.4. Expression of NAT1 and NAT2 intracellular proteins on $CD3^+$ or $CD19^+$ lymphocytes by flow cytometry

The percentage of lymphocytes expressing the intracellular proteins of interest was evaluated using monoclonal antibodies. First, PBMC from patients and the control group were labeled with anti-CD3-PE or anti-CD19-FITC (eBiosciences®, CA, USA) for 20 min. Then, the cells were treated with the commercial Fix/Perm Buffer Kit (eBioscience) and incubated with rabbit anti-NAT1 or mouse anti-NAT2 antibodies (primary) (Abcam, Cambridge, UK) for 1.5 h at 4°C. Later, the cells were incubated with anti-rabbit APC or anti-mouse APC secondary antibodies (eBioscience), respectively, for 20 min at 4°C in the dark. Afterwards, cells were fixed with 1% paraformaldehyde,

and the percentage of double-positive cells was obtained via a FACS Canto II Cytometer and analyzed using FlowJo V10.6.1 software (LLC, BD®). The results were expressed as the percentage of positive cells by NAT1 or NAT2. In order to represent the expression of NAT1 and NAT2 in absolute values, the hematic biometry data of all the participants were used.

2.5. *In situ* arylamine N-acetyltransferase assay

PBMC (2×10^5 cells) from patients and the control group were cultured in RPMI 1640 medium supplemented with 50 U/mL penicillin and 50 µg/mL streptomycin (Sigma-Aldrich), and maintained at 37°C in a humidified atmosphere of 5% CO_2; the medium contained the specific substrates 100 µM acid para aminobenzoic (PABA) (Sigma–Aldrich) or isoniazid (INH) (Sigma–Aldrich) for each enzyme (26, 27). The cells were incubated for 24 h; after this time, the supernatant was removed and frozen at -80°C until HPLC analysis.

2.6. *In situ* NAT enzymatic activity determination

NAT1 and NAT2 metabolic activities were determined by HPLC on supernatants from PBMC cell cultures of patients and the control group (100 µL) to quantify the concentrations of the substrates and metabolites for each enzyme: PABA and acetyl-PABA (AcPABA) for NAT1, INH and acetyl-INH (AcINH) for NAT2. For the extraction step, the supernatants were dried in the concentrator plus/Vacufuge® Eppendorf® plus (Hamburg, Germany) at room temperature. The residues were reconstituted in 100 µL of 80% mixture composed of 50mM acetic acid and 20% acetonitrile for NAT1 and 80% of 20 mM 1-heptanesulfonic acid sodium, 2.5 mM phosphate buffer (pH= 2.87), 10% ACN and 10% trichloroacetic acid at 10% (w/v) for NAT2. After centrifugation at 900 x g for 3 min at 4°C, a 20 µL aliquot of the solution was injected into the HPLC system. The Waters HPLC chromatographic equipment consists of a 1525 binary pump system linked to a 717 Plus autoinjector with a 20 µL injection loop, a UV/VIS 2487 detector, and Breeze software v3.2 (WatersCorporation, MA, USA). The guard column was an X-Terra Shield RP18, 125 A, 5 µm, 3×20 mm (Waters Corporation, MA, USA). The

standards and chromatographic solvents were HPLC grade. The mobile phase consisted of 80% 50 mM acetic acid, 20% acetonitrile with a wavelength of 270 nm for NAT1, and 97% 20 mM 1-heptanesulfonic acid sodium, 2.5 mM phosphate buffer (pH = 2.87), 3% acetonitrile, with a wavelength of 266 nm for NAT2; at a constant flow of 0.4 mL/min in both cases.

The methods described were optimized from a work previously carried out by our research group (26) and were analytically validated according to the International conference on harmonization (28) . The concentration of each analyte in the sample was calculated using the respective calibration curve. PABA and INH N-acetylation was determined by measurement of nanomoles of AcPABA over 24 h per mL and nanomoles of AcINH over 24 h per mL, respectively.

2.7. Barnes-Hut t-Distributed Stochastic Neighbor Embedding analysis

To display subpopulations of interest ($CD3^+$, $CD19^+$) and marker expression, we performed heat maps ($NAT1^+$, $NAT2^+$) of PBMC from the participants. Barnes-Hut t-Distributed Stochastic Neighbor Embedding (t-SNE) by FlowJo V10.6.1 implementation with next parameters (perplexity= 50, θ= 0.5, interactions= 1000, Euclidean distance) was performed (29, 30). For this analysis, we classified the patients as relapse in ALL and those with their first diagnosis. Events from 6 samples of each group were concatenated and down-sampled, providing a common t-SNE map distribution, allowing the sample comparison.

2.8. PCR amplification of NAT1 and Sequencing study

Total DNA from patients and controls was isolated using a Wizard® Genomic DNA Purification Kit (Promega Corporation). To determine the presence of SNP 559 C>T (rs5030839) and 560 G>A (rs4986782) from NAT1, we amplified the region of interest, using the Phusion® High Fidelity DNA polymerase, New England BioLabs®, and the primers Fw 5'-CCATTGATGGCAGGAACTACA-3' and Rv 5'-GATAACTGGTGAGCTGGATGAC-3'. The thermocycling conditions were as follows: 98°C for 30 s, 35 cycles of 98°C for 10 s, 64°C for 20 s, 72°C for 30 s, followed by a

final extension 72°C for 7 min. The amplicons obtained were purified using minelute® reaction cleanup kit (Qiagen) and were sequenced by the Sanger method, in an equipment AB 3130 at the Instituto Potosino de Investigación Científica y Tecnología (IPICYT).

2.9. Chromatin immunoprecipitation assay

PBMC were isolated and treated with 1% formaldehyde to cross-link the chromatin and later sheared at 45% amplitude for 12 cycles of 30 seconds ON and 30 seconds OFF in the EpiSonic™ 2000 Sonication System, to obtain DNA fragments to approx. 300-700 pb. The crosslinked protein-DNA complexes were immunoprecipitated at 4°C overnight using magnetic beads (Dynabeads Protein G, Thermofisher) and the antibodies were anti-histone H3 (Abcam® ab1791), anti-histone H3 trimethyl K27) (Abcam® ab6002), and anti-acetyl histone H3 (K14) (Merck Millipore®). Then, the cross-links were reversed and the immunoprecipitated DNA was purified and amplified using the kit Phusion® High Fidelity DNA polymerase, New England BioLabs®. The thermocycling conditions were 98°C for 30 s, 35 cycles of 98°C for 10 s, 64°C for 20 s, 72°C for 30 s, followed by a final extension at 72°C for 7 min. The primers to study the promoter were Fw 5'-CACCAGCATAAACAAAGCCATA-3' and Rv 5'-AAGCAGAACTGGTAACCTAGAG-3' for NAT1; and Fw 5'-AGAGGACAGAAATCTGGC AG-3' and Rv 5'-TGATTGCCTCCTACTCCTGG-3' for NAT2.

2.10. Statistical Analysis

Normality tests of the requested data were performed; in the case of parametric data, the means were compared using the Student's T- test. Medians were tested with the Mann-Whitney U test if the data were non-parametric. The level of statistical significance was $p<0.05$. Statistical tests were performed using GraphPad Prism V 7.00 (GraphPad Software Inc., La Jolla, CA).

3. Results

3.1. Study groups

The demographic and anthropometric characteristics of the study groups are described in Table 1. The distribution of the body mass index (BMI) was similar between both groups and most participants were normoweight. 90% of ALL patients were in the maintenance phase. 7 out of 20 patients with ALL had a relapse, 4 at the bone marrow level and 3 at the central nervous system level. In addition, 3 relapses were reported due to discontinuation of the treatment and the other 4 due to an unknown cause.

3.2. NAT1 and NAT2 protein and mRNA expression in PBMC from ALL patients

To determine if there was a difference in NAT1 and NAT2 mRNA expression between control subjects and patients with ALL, we carried out RT-PCR analysis. We found low levels of NAT1 mRNA in the group of patients with ALL compared to the control group (Fig. 1A, $p=0.001$). In contrast, NAT2 mRNA expression was similar between both groups (Fig. 1B, $p=0.51$).

NAT1 and NAT2 protein, measured by flow cytometry, indicated similar percentages of $NAT1^+$ or $NAT2^+$ lymphocytes between controls and patients. However, there was a higher percentage NAT1 positive cells in both groups studied (Fig. 1C) as compared to NAT2 positive lymphocytes (Fig. 1D). It is interesting to notice that there was a high heterogeneity in the expression of NAT1 and NAT2 protein in lymphocytes from the studied subjects. Thus, we decided to analyze the expression of these proteins in subpopulations of T lymphocytes ($CD3^+$) and B lymphocytes ($CD19^+$). Our results showed a low expression of $CD19^+$ lymphocytes in patients with ALL compared to the control group ($p=0.0001$); however, it is important to note that patients were undergoing chemotherapy when the sample was taken (data not shown), thus the cytotoxic activity of the drugs used influenced the decrease of this cellular subpopulation.

Subsequently, a double positive cell analysis was performed to identify T lymphocytes positive for NAT1 or NAT2 ($CD3^+/NAT1^+$ and $CD3^+/NAT2^+$). Although a high variability of the data was observed, the percentage of $CD3^+/NAT2^+$ cells was higher in ALL

patients compared to the control group (p=0.04) (Fig. 1F). In contrast, there was no difference in the percentage of $CD3^+/NAT1^+$ cells between the two groups. Interestingly, low levels of NAT1 expression were observed in 5 out of the 7 patients who had relapsed (Fig. 1E).

Due to the high variability in the data obtained with the percentage of positive cells, using the hematic biometry data of each participant from the clinical record we calculated the absolute values of NAT1 and NAT2 as well as the double positive cells ($CD3^+/NAT1^+$, $CD3^+/NAT2^+$) (Fig. 2A-D). Low levels of NAT1 expression in patients with ALL (p=0.0003) (Fig. 2a), as well as in $CD3^+/NAT1^+$ double positive cells (p=0.0049) (Fig. 2C), were confirmed, as compared to control group. The absolute value of NAT2 (Fig. 2B) or $CD3^+NAT2^+$ cells (Fig. 2D) did not show any significant differences between the studied groups.

3.3. In situ enzymatic activity of NAT1 or NAT2 in cultures of blood mononuclear cells

Due to the low expression observed at the protein level in lymphocytes, we analyzed the enzymatic activity of NAT1 and NAT2. A decreased NAT1 activity was observed in most patients with ALL as compared to the control group (p=0.0047) (Fig. 3A), possibly related to the lower levels of NAT1 previously described (absolute values). There was no difference in the activity of NAT2 between the studied groups (Fig. 3B). In addition, to evaluate if the enzymatic activity of these proteins had an influence on relapse in ALL, we determined the activity in PBMCs from patients with this condition. However, no differences were found between patients with relapses and controls (Fig. 3C and D).

3.4. Correlations analysis between NATs levels, enzymatic activity, and BMI

Statistical tests of correlation between mRNA expression, absolute values, percentage of positive cells, and the enzymatic activity of NAT1 and NAT2 were performed in the study groups; however, no statistically significant results were observed (data not shown). Statistically significant results were found in the control group when correlating

BMI with the percentage of positive cells, (Pearson, r=-0.6, p=0.01). Nevertheless, no correlation was observed in patients with ALL (Pearson, r=-0.09, p=0.71); in this sense, a correlation was observed between the BMI and the absolute values of NAT1 in the control group (Spearman, r=-0.48, p=0.046) but not in patients (Pearson, r=0.15, p=0.62). However, the influence of BMI on the expression at the protein level of NAT1 in healthy children is not clear and the reason for the inversely proportional behavior is unknown.

3.5. t-SNE map

The t-SNE method creates a two-dimensional map from a reduction of parameters, grouping the data by common parameters generating clusters; when these clusters are more separated, more differences between them have been demonstrated. The heatmap showed the expression of our markers and highlighted the distribution of the marker of interest. Figs. 4 and 5 in the first column (4A,C,E; 5A,C, and E) show the regions corresponding to the $CD3^+$ and $CD19^+$ subpopulations of the control group, the group of patients at first diagnosis, and the group of patients in relapse.

The t-SNE map demonstrated the expression of $CD3^+$ lymphocytes in patients with relapse was concentrated in a single cell island (Fig. 4E), meanwhile for patients at their first diagnosis, 3 islands were present (Fig. 4C). In the control group, 2 regions with high expression levels of $CD3^+$ were observed (Fig. 4A). The analysis of $CD19^+$ cells showed low levels of CD19+ in patients with relapses (Fig. 4E) compared to the control group (Fig. 4A). In the group of patients with the first diagnosis of ALL, no expression of $CD19^+$ subpopulation was observed (Fig. 4C).

Fig. 5A, C and E present the distribution of the subpopulations $CD3^+$ and $CD19^+$ in the groups. The representation of the subpopulations is a little different as the t-SNE methodology is not reproducible (29, 31). It is worth mentioning that the same data from the patients presented in Fig. 4A, C and E were used.

The analysis of the expression of NAT1 in $CD3^+$ and $CD19^+$ cells showed the presence of this enzyme in both subpopulations of lymphocytes in the control group (Fig. 4B),

however, NAT1+ cells were also detected in a third island that did not correspond to the cells of interest ($CD3^-CD19^-$). In $CD3^+$ lymphocytes from patients with first diagnosis and relapse, a low expression of NAT1 was observed, however in the unidentified cells ($CD3^-CD19^-$) the expression was high (Fig. 4D and F).

A high expression of NAT2 was observed in $CD3^+$ cells from patients with first diagnosis and relapse (Fig. 5D and F) compared to the control group (Fig. 5B). This finding is consistent with our analysis of $CD3^+/NAT2^+$ (Fig. 1F). On the other hand, the analysis of NAT2 expression in the $CD19^+$ subpopulation showed that NAT2 was not present in the entire $CD19^+$ region in the control group, since it was only concentrated in a small area (Fig. 5B). In patients with relapses, a low expression was observed in this region with respect to the control group (Fig. 5F). Interestingly, and in a similar manner to the NAT1 expression, a high expression of NAT2 was detected in the group of unidentified cells ($CD3^-CD19^-$), both in patients at first diagnosis and with relapses (Fig. 5D and F).

3.6. Sequencing analysis

The low or lack of activity of NAT1 detected by HPLC in some patients, it prompted us to examine the possible presence of SNPs in the NAT1 gene in these subjects. We evaluated by sequencing the presence of the SNP 559 C>T (haplotype *NAT1*15)* and 560 G>A (haplotype *NAT1*14B*). We did not detect SNP 559 or 560 in any of the 5 patients who had a low NAT1 enzymatic activity. This group of patients were wild homozygous (C,C by 559) (G,G by 560) (Fig. 6). Therefore, the influence of these SNPs on the enzymatic activity of NAT1 was ruled out.

3.7. Chromatin immunoprecipitation

To examine whether the low expression of mRNA and protein of NAT1 observed in ALL patients, was related to alterations in histone acetylation and methylation on the NAT1 gene, a pilot chromatin immunoprecipitation test was performed. The assay was also performed for NAT2 because some patients had a low expression at the same molecular levels.

The results indicated that lysine 14 acetylation in histone H3 (H3K14Ac) was decreased in the promoter of the NAT1 gene in PBMC from the patients with ALL compared to the control subjects (Fig. 7A and B). In contrast, the modification of trimethylation of histone H3 in lysine 27 (H3K27me3) could not be detected in the selected region of the promoter, so we sought to analyze another region of histones, such as H3K9me3 or H3K4me3 in the same promoter. It is important to mention that NAT1 mRNA has 2 isoforms and we evaluated isoform A. The ENCODE® database through UCSC Genome Browser (https://genome.ucsc.edu/) was used to detect a region that had a big interaction of histones with the promoter of NAT1, specifically with H3K4me3. The amplicons of NAT1 (202 bp) and NAT2 (171 bp) were observed under the different immunoprecipitation conditions (Input, anti-histone H3 control, anti-histone H3 acetyl K14, and anti-histone H3 trimethyl K27) (Fig. 7).

When the modification H3K14Ac and H3K27me3 in the NAT2 gene were analyzed, we found hypoacetylation and hypermethylation in the NAT2 promoter in ALL patients compared to the control group (Fig. 7C and D); however, no changes in mRNA expression were observed (Fig. 1B). Therefore, it is not clear with our results how these alterations of histones influence NAT2 expression.

4. Discussion

The accumulation of active carcinogenic metabolites could be involved in the development of some types of cancer, including ALL. Therefore, xenobiotic enzymes with a high metabolic activity such as NATs could have an important role in this neoplasm. The presence of several SNPs of these enzymes and the impact on the likelihood of developing ALL is well described through genetic association studies (3, 11, 14, 15, 32). To our knowledge, there are no other studies that have explored the expression and enzymatic activity of N-acetyltransferases in patients with ALL.

The present study demonstrated that blood lymphocytes from patients with ALL show a lower expression of NAT1 at the mRNA level, in terms of absolute values compared to the control group, while for NAT2 the result was similar between both groups. The

correlation analysis showed no significant correlations between the enzymatic activity, the expression at mRNA or the protein level of each gene, unlike the study by Salazar-González et al (27). However, we did not determine the genotype of each participant, which could explain why in our study no correlations were observed with the variables of interest.

In addition, our data showed that NAT1 activity is decreased in LLA patients, which is in accordance with previous studies in leukemia cell lines (THP-1, Jurkat, or CEM) as well as in liver (HepG2) a and colon cancer cell line (HT-29) (33). However, a high NAT1 activity has been reported in breast cancer (T-47D, ZR-751) and prostate cancer (LNCaP, 22RV1) cell lines (33). The mechanism for this opposing behavior of NAT1 among different cancers is unknown, although post-translational modifications or epigenetic mechanisms could be involved (33).

In the analysis of $NAT1^+$ lymphocytes, specifically in the percentage of $CD3^+$ cells, two clear zones were present: those with high or low levels. It was notable that the $CD3^+/NAT1^+$ low expression group included the patients who had relapsed (Fig. 1E). Also, in the t-SNE analysis, we observed that the level of NAT1 expression was low in $CD3^+$ lymphocytes in the group of patients with relapses. The patients with ALL in this study were type B, thus, we expected to find alterations in the expression of NAT1 in $CD19^+$lymphocytes and we hypothesized these alterations could have had an influence on the likelihood of relapse. Unfortunately, as the patients expressed low numbers of B lymphocytes, the analysis of NAT1 in $CD19^+$ lymphocytes was difficult to carry out.

During the recruitment process, a blood sample was taken from a patient diagnosed with relapse but still without cancer treatment. In this particular patient, there was a high expression of $CD19^+$ lymphocytes without expression of NAT1, as well as null enzyme activity. These results suggest that NAT1 might have an implication in the development of the neoplasm, but it is necessary to evaluate the expression in $CD19^+$ lymphocytes in untreated patients. So, we think that the low expression of NAT1 in $CD3^+$ or $CD19^+$ lymphocytes could be used as a poor prognostic factor.

Salazar-González et al. (27) studied the expression of NAT1 in PBMC from healthy adults, among lymphocytes T ($CD3^+$), B ($CD19^+$), and NK ($CD56^+$) cells, and demonstrated that the expression of NAT1 was higher in T lymphocytes compared to other lymphocyte subpopulations, with a wide range of expression (15%-80%). Those values were similar to those observed in our control group (18%-90%). Meanwhile, the low levels of NAT1 in $CD19^+$ lymphocytes (2%-4%) observed previously (27), are in contrast to our study, as there was a high variability in expression levels in the control group (21%-80%) and also the age of the populations was distinct between both studies.

For the specific case of the null or low activity of NAT1, one of our hypotheses was that this occurred due to the presence of the SNPs 559 C>T (haplotype *NAT1*15*) and 560 (haplotype *NAT1*14B*). The first SNP was characterized by generating a truncated protein and the second one for a protein with a slow acetylator phenotype (http://nat.mbg.duth.gr/). However, none of the patients with a low activity of NAT1 presented with these SNPs, which could indicate that post-translational modifications could influence the enzyme activity or that there are some other SNPs or mutations that generate a truncated or non-functional protein. Two patients with relapses were in this genotyping trial. As mentioned above, the ALL patients were sampled when they were undergoing chemotherapy, which consisted mainly of the administration of mercaptopurine, methotrexate, and L-asparaginase, according to the Mexican clinical practice guidelines(20). However, none of these drugs is a substrate or inhibitor for NAT1 or NAT2 (34). Therefore, it is necessary to perform molecular studies to explain if the decreased NAT1 enzymatic activity participates in the carcinogenesis process and determine, in particular, its impact on the regulation of p53 (16) or the consequences of the alteration of the concentrations of acetyl coenzyme (18, 35).

In comparison with our study (control group 6.65 nmol/24h/mL; patients with ALL 6.9 nmol/24h/mL), in the case of the enzymatic activity of NAT2, a greater acetylation of INH in PBMC from healthy subjects (22 nmol/min/mg protein) or HeLa cells (40 nmol/min/mg protein) (26) has been previously reported. However, it is worth

mentioning the activity was evaluated previously in subjects older than 20 years. Thus, our data can be explained because there is an immaturity in the synthesis process of this enzyme in healthy children. Additionally, the enzymatic activity of NAT2 was not different between the study groups despite the differences at the protein level. Several factors can explain these results. Firstly, the activity test was performed in PBMC and not in subpopulations and, secondly, SNPs might have been present that generate a slow phenotype despite high levels of protein expression.

In the analysis of $NAT2^{+}$ lymphocytes, the results obtained in $CD3^{+}$ cells (percentage of positive cells) from patients with ALL were interesting (Fig. 1F), because we expected that patients with ALL would show a low expression, since NAT2 is the enzyme that performs the N-acetylation, which provides a protective effect for the development of cancer (3, 13). However, ALL patients presented with a higher expression compared to the control group, despite a lack of differences at the mRNA level. The t-SNE analysis demonstrated that the first diagnosis patients showed higher levels of NAT2 expression in $CD3^{+}$ lymphocytes than patients with relapses or the control group. Therefore, at present, the function of NAT2 in these T lymphocytes from ALL patients is not known. Further experiments need to be performed to elucidate the possible role of NAT2 in this specific population.

The changes in the expression of the genes in eukaryotic cells are controlled by epigenetic mechanisms and their aberrations can contribute to the development of cancer (36, 37). Thus, we determined the status of acetylation and methylation of histones in the promoters of NAT1 and NAT2 genes. First, a computer analysis was performed using the Encode® database through the UCSC Genome Browser (https://genome.ucsc.edu) and Washu epigenome Browser (http://epigenomegateway.wustl.edu/legacy/) to find out which regions of the NAT1 and NAT2 promoter show the highest interaction with Histone H3. In the case of the NAT1 promoter in isoform A, a strong interaction was found with histone acetylation of H3 lysine 27 (H3K27Ac) as well as H3K4me3, H3K9me3 in leukemia cell lines (Jurkat, GM12878). In contrast, there are no reports for the isoform B of promoter regions

having strong interaction with post-translational modifications; for the NAT2 promoter there was found to be a strong interaction with H3K4m3 in the same cell lines.

Thus, we decided to evaluate the interaction with other regions of histones that have not been reported. Based on our preliminary results from the CHIP assay, we found a diminution in H3K14Ac in the promoter of NAT1 of patients with ALL compared to controls. Therefore, this modification may play a key role in the adequate activation of the transcription of the NAT1 gene in healthy PBMC. In agreement with our results, it has been reported that the acetylation of histone H3 and H4 in PBMCs from patients with ALL and acute myeloid leukemia (AML) was deficient when compared with healthy adults (38).

Unlike in the control group, we found acetylation and methylation in the promoter of NAT2 in patients with ALL. Our results concur with those reported by Yong Zou et al. (39), as they observed aberrant methylation in histone H3 in PBMC from patients with ALL and AML when compared with healthy adults. However, we do not know the influence of these modifications on this promoter, due to the fact that no changes were observed at the mRNA level. It is possible that performing this analysis at the subpopulation level of lymphocytes would allow us to find a correlation between the cellular subpopulation and enzymatic activity and to deduce that NATs have an impact on the progression of disease and these variables could be used as prognostic factors. Although we studied other acetylated and methylated regions of histone H3, all the results indicated that the promoter of the NAT1 isoform A and NAT2 have too much interaction with the stem sites of histone H3.

In conclusion, the low mRNA expression level and absolute value of NAT1 as well as its low/null enzymatic activity can influence the carcinogenesis process and relapses of this neoplasia. However, the high levels of NAT2 expression observed in $CD3^{+}$ cells of patients with ALL might be not involved in the abnormalities present in this condition. Alterations in the post-translational modifications of histones were observed, both in the promoters of NAT1 (in isoform A) and of NAT2, although only alteration in the expression of the NAT1 messenger was observed.

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Conflict of interest

The authors declare that they have no conflicts of interest.

Compliance with ethical standards

All persons gave their informed consent prior to their inclusion on the study. The study was performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments.

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Table 1. Demographic and anthropometric data of the study groups, values presented with median (range from minimum to maximum).

	CONTROL GROUP	**PATIENTS**
n	19	20 (19 HR, 1 LR), (7 relapses)
Sex	10 men 9 women	12 men 8 women
Age (years)	8 (4-15)	8.5 (3-15)
Weight (Kg)	37 (15-82)	35 (10.8-66)
Height (m)	1.4 (1.1-1.74)	1.3 (0.88-1.63)
BMI (Kg/m^2)	21 (13.3-29) 3 underweight, 10 normoweight 1 overweight, 5 wereobese	20.17 (8.8-29.3) 2 underweight, 9 normoweight 3 overweight, 6 wereobese
Concomitant diseases	1 Gastroenteritis 1 Kidney stones	1 Down's Syndrome 1 Hyperthyroidism
Treatment stage	----	17 Maintenance treatments, 1 Consolidation therapy, 2 Reinduction chemotherapy
EGIL	-----	2 Egil B1, 9 Egil B2 9 Egil B3

BMI: body index mass; HR: High-risk; LR: Low-risk; EGIL: European Group for the Immunological Characterization of Leukemias, B linage: B1 ALL pro-B, B2 ALL common, B3 ALL pre-B.

Fig.1.

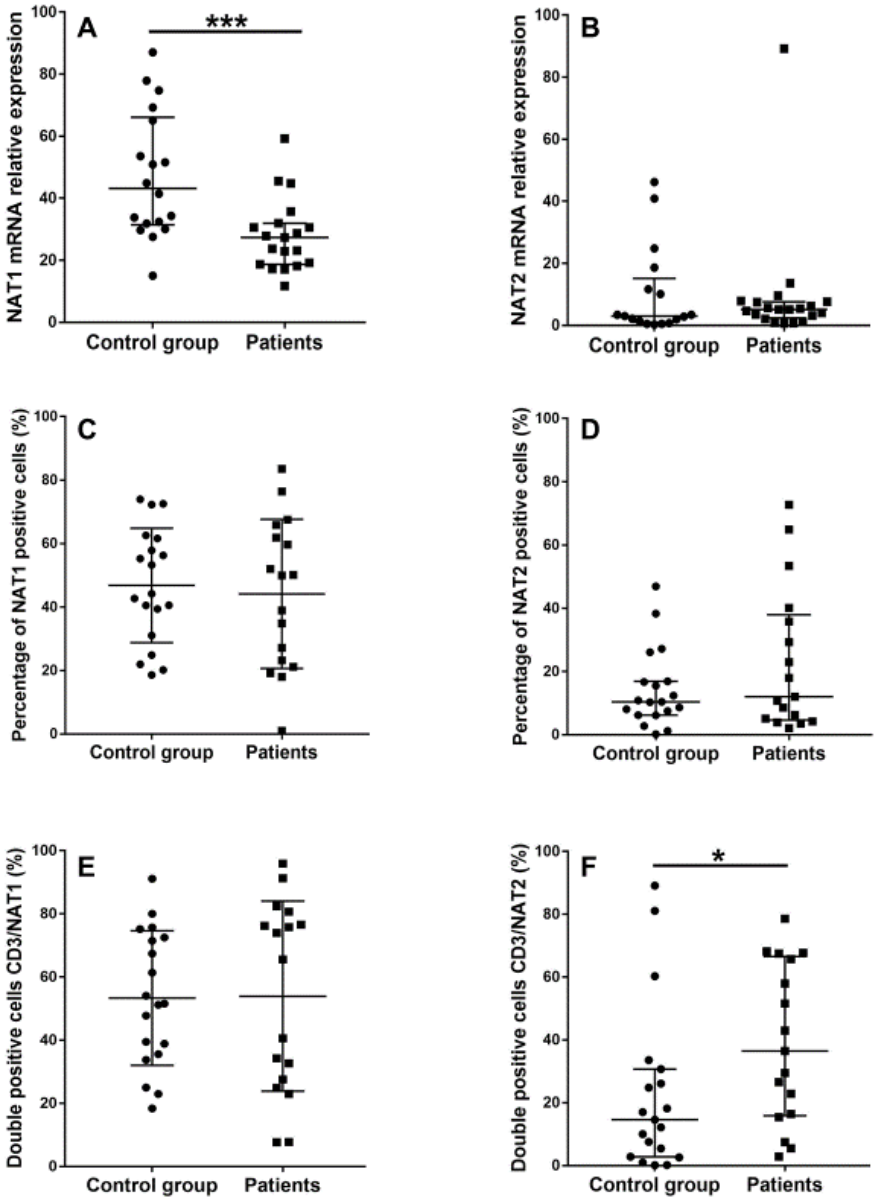

A
***
NAT1 mRNA relative expression
Control group
Patients
B
NAT2 mRNA relative expression
Control group
Patients
C
Percentage of NAT1 positive cells (%)
Control group
Patients
D
Percentage of NAT2 positive cells (%)
Control group
Patients
E
Double positive cells CD3/NAT1 (%)
Control group
Patients
F
*
Double positive cells CD3/NAT2 (%)
Control group
Patients

Fig. 2.

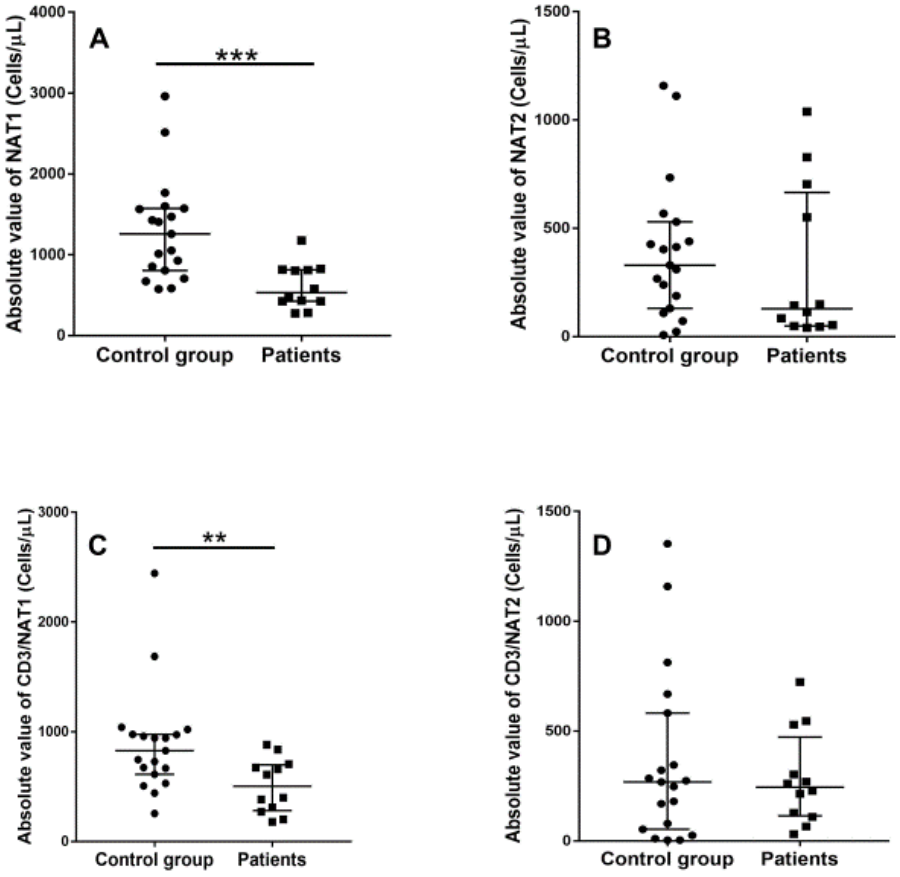

A
***
Absolute value of NAT1 (Cells/μL)
4000
3000
2000
1000
0
Control group
Patients
B
Absolute value of NAT2 (Cells/μL)
1500
1000
500
0
Control group
Patients
C
**
Absolute value of CD3/NAT1 (Cells/μL)
3000
2000
1000
0
Control group
Patients
D
Absolute value of CD3/NAT2 (Cells/μL)
1500
1000
500
0
Control group
Patients

Fig. 3.

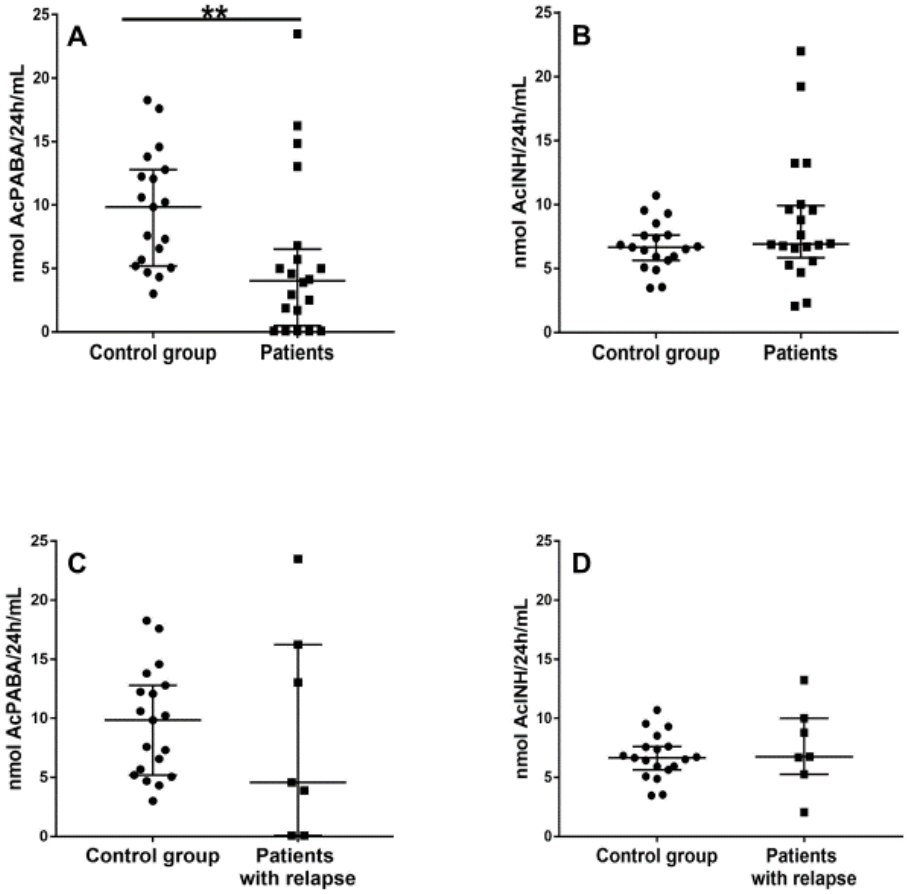

A
**
nmol AcPABA/24h/mL
25
20
15
10
5
0
Control group
Patients
B
nmol AcINH/24h/mL
Control group
Patients
C
nmol AcPABA/24h/mL
Control group
Patients with relapse
D
nmol AcINH/24h/mL
Control group
Patients with relapse

Fig. 4.

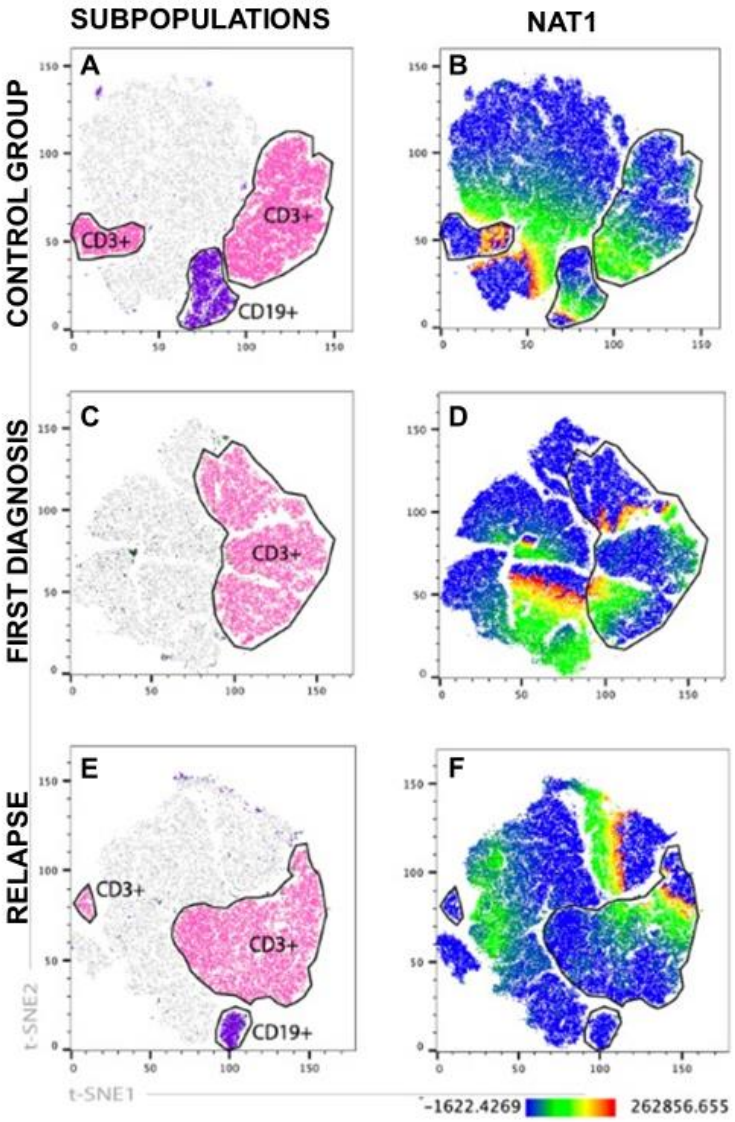

SUBPOPULATIONS
NAT1
CONTROL GROUP
FIRST DIAGNOSIS
RELAPSE
A
B
C
D
E
F
CD3+
CD19+
t-SNE2
t-SNE1
-1622.4269
262856.655

Fig. 5.

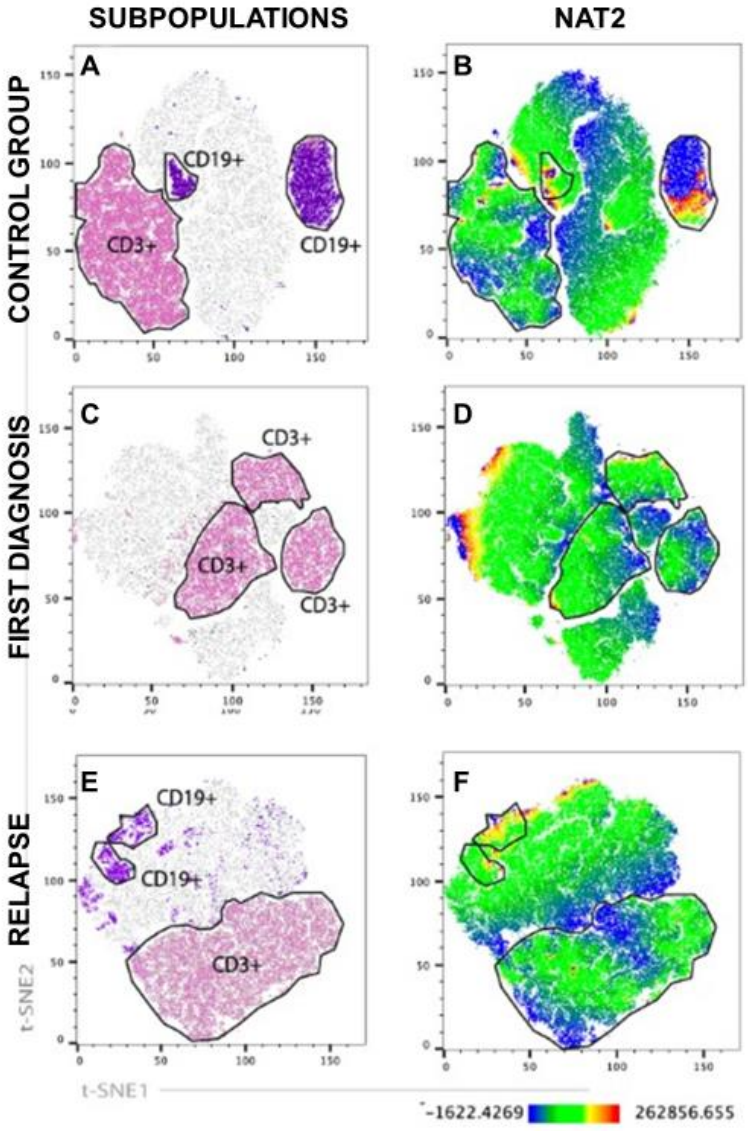

SUBPOPULATIONS
NAT2
CONTROL GROUP
FIRST DIAGNOSIS
RELAPSE
A
B
C
D
E
F
CD19+
CD3+
CD19+
CD3+
CD3+
CD3+
CD19+
CD19+
CD3+
t-SNE2
t-SNE1
-1622.4269
262856.655

Fig. 6.

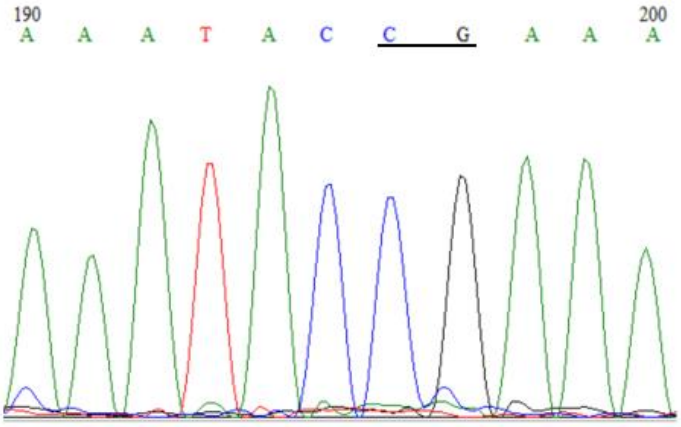
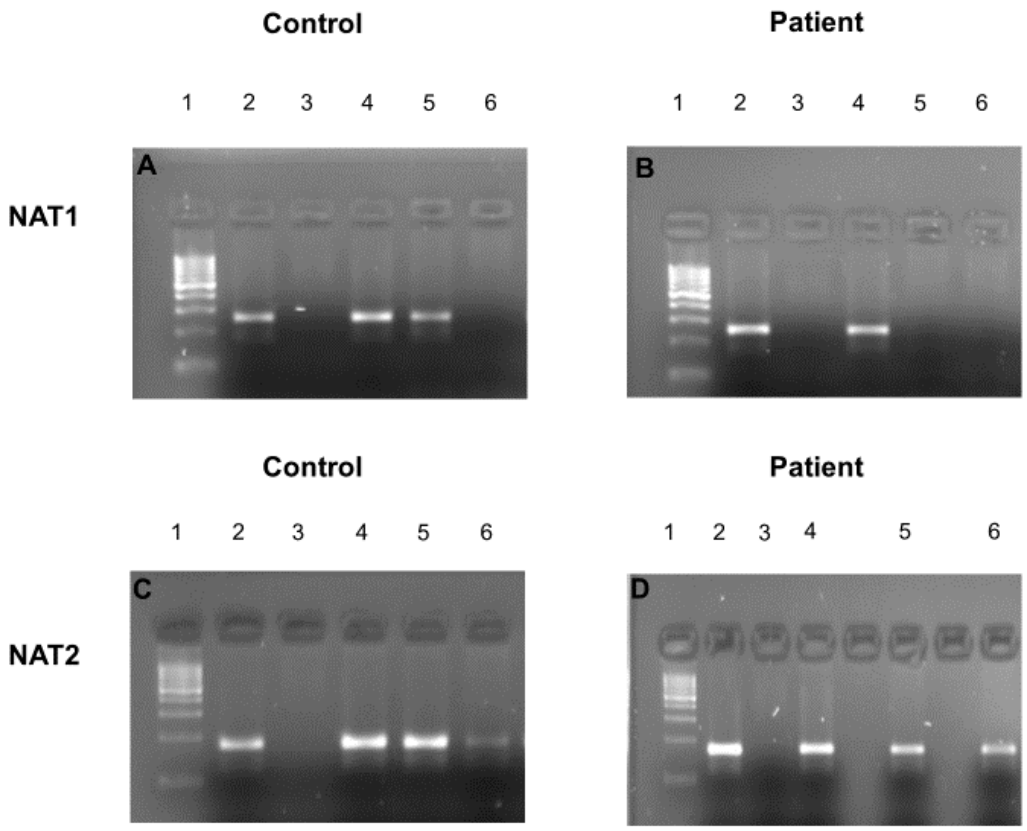


Fig. 7.


Control
Patient
1 2 3 4 5 6
1 2 3 4 5 6
A
B
NAT1
Control
Patient
1 2 3 4 5 6
1 2 3 4 5 6
C
D
NAT2

7

Figure Legends

Fig. 1. NAT1 and NAT2 expression at the level of mRNA and protein.

a Relative expression of NAT1 mRNA in patients (n=19) (median; interquartile range) (27.36; 18.69-31.97) and control group (n=18) (43.19; 31.41-66.11), Mann Whitney U test was employed. **b** Relative expression of NAT2 mRNA in patients (n=20) (5.11; 2.37-7.57) and control group (n=17) (2.98; 1.05-15.12). **c** Percentage of $NAT1^+$ lymphocytes from patients (n=17) (44.1 ± 23.5) (mean ± Standard deviation) and control group (n=19) (46.8 ± 18). **d** Percentage of $NAT2^+$lymphocytes from patients (12; 4.6-37.9) (n=17) and control group (n=19) (10.4; 6.2-16.9). **e** Percentage of positive cells ($CD3^+/NAT1^+$) from patients (n=17) (53.9 ± 30) and control group (n=19) (53.3 ± 21.2). **f** Percentage of positive cells ($CD3^+/NAT2^+$) from patients (36.5; 15.9-66.6) (n=17) and control group (n=19) (14.6; 2.8-30.7). The Mann Whitney U test was employed.

Fig. 2. Absolute values of NAT1 and NAT2 in the control group and patients with ALL.

a The absolute values of NAT1 from patients (n=12) (median; interquartile range) (532; 424-815) and control group (n=19) (1257; 806-1574), Mann Whitney U test was employed. **b** The absolute values of NAT2 from patients (n=12) (129; 49.5-665) and control group (n=19) (329; 130-530). **c** The absolute values of $CD3^+/NAT1^+$ from patients (n=12) (median; interquartile range) (504; 281-698) and control group (n=19) (827; 612-976). **d** The absolute values of $CD3^+/NAT2^+$ from patients (n=12) (245; 114-473) and control group (n=19) (268; 53-582). The student t-test was employed.

Fig. 3. Enzymatic activity of NAT1 and NAT2 in control group, patients with ALL and relapse in ALL exclusively.

a The enzymatic activity of NAT1 between patients (n=20) (4; 0.48-6.52) and control group (n=19) (median; interquartile range) (9.84; 5.2-12.8), Mann Whitney U test was employed. **b** The enzymatic activity of NAT2 from patients (n=20) (6.9; 5.84-9.91) and control group (n=19) (6.65; 5.63-7.61). **c** The enzymatic activity of NAT1 from patients with relapses (n=7) (4.58; 0.08-16.24) and control group (n=19) (9.84; 5.2-12.8). **d** The enzymatic activity of NAT2 from patients with relapses (n=7) (6.75; 5.26-10) and control group (n=19) (6.65; 5.63-7.61). Mann Whitney U test was employed.

Fig. 4. t-SNE analysis of NAT1 in $CD3^+$ and $CD19^+$ cells.

t-SNE projections of six samples of lymphocytes with NAT1 from each group, Control, ALL first time, and ALL relapse. Lymphocytes have been coded according to fluorochrome staining ($CD19^+$: Purple; $CD3^+$: Pink) (**a,c** and **e**). Heatmap has been color coded according to the expression level of the marker, as indicated (**b, d** and **f**) (Blue: low; Red: high). t-SNE parameters (perplexity = 50, θ = 0.5, iterations = 1000, Euclidean distance).

Fig. 5. t-SNE analysis of NAT2 in $CD3^+$ and $CD19^+$ cells.

t-SNE projections of six samples of lymphocytes with NAT2 from each group, Control, ALL first time, and ALL relapse. Lymphocytes have been coded according to fluorochrome staining ($CD19^+$: Purple; $CD3^+$: Pink) (**a,c** and **e**). Heatmap has been color coded according to the expression level of the marker, as indicated (**b, d** and **f**) (Blue: low; Red: high). t-SNE parameters (perplexity = 50, θ = 0.5, iterations = 1000, Euclidean distance).

Fig. 6. Sequencing analysis.

Example of direct sequencing chromatogram. SNPs 559 C>T (haplotype *NAT1*15,* truncated protein/no enzyme activity) and 560 G>A (haplotype *NAT1*14B*, slow activity) were not observed in the *NAT1* gene.

Fig. 7. Histone H3 acetylation and methylation in peripheral blood mononuclear cells.

Agarose gels (3%) of the amplicons of immunoprecipitated chromatin (CHIP) of *NAT1* (202 bp) and *NAT2* (171 bp) in a control subject (**a** and **c**) and a patient with ALL (**b** and **d**). Agarose gels were stained in ethidium bromide and run at 100V for 30 min at room temperature. *Lane 1* 100-1000 bp DNA ladder (Jena Bioscience®), *lane 2* Input, *lane 3* negative, *lane 4* Anti-Histone H3 control, *lane 5* Anti- Histone H3 acetyl K14, *lane 6* Anti-Histone H3 trimethyl K27.

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ORIGINAL ARTICLE: RESEARCH

Genetic polymorphisms of arylamine N-acetyltransferases 1 and 2 and the likelihood of developing pediatric acute lymphoblastic leukemia

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ABSTRACT

Acute lymphoblastic leukemia (ALL) is one of the main causes of death in children and is associated with both genetic susceptibility and environmental factors. Genes encoding the arylamine N-acetyltransferases 1 and 2 *(NAT1* and *NAT2)* isoenzymes are highly polymorphic among populations. Single-nucleotide polymorphism analysis was performed by real-time polymerase chain reaction from the genomic DNA of 225 healthy subjects and 57 children with ALL diagnoses. Significant associations were found between the development of ALL and the presence of the haplotypes *NAT1*3* (Odds ratio [OR], 2.1), *NAT1*4* (OR, 1.92*), NAT2*6B* (OR, 3.30), *NAT2*6J* (OR, 3.25) and *NAT2*7A* (OR, 2.45) and the NAT1 rapid (OR, 6.69) and NAT2 slow phenotypes (OR, 2.95). Our results indicate that haplotypes that provide rapid NAT1 and slow NAT2 acetylating phenotypes may influence the development of ALL in children.

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Arylamine N-acetyltransferases; NAT1 and NAT2; SNP; acute lymphoblastic leukemia

Introduction

Acute lymphoblastic leukemia (ALL) is the most common malignancy diagnosed in pediatric patients and represents 25–30% of all types of malignancies in children [1–4]. The reported incidence of ALL in Latin America is higher than in other regions of the world [5]. The prevalence of ALL cases in Mexico between 2007 and 2012 was 5864, accounting for 83% of all malignancies in pediatric patients [6]. In 2012, 89 new cases were diagnosed, with an incidence rate of 44.5 per million people [6,7]. Although the etiology of ALL is unknown, genetic and environmental factors appear to be involved [8]. Single-nucleotide polymorphisms (SNPs) of enzymes involved in xenobiotic metabolism, such as arylamine N-acetyltransferases (NAT) NAT1 and NAT2 [3,9–12], could be involved in the likelihood of developing ALL.

The genes *NAT1* and *NAT2* are located on chromosome 8 (*NAT1* at 8p21.3-23.1 and *NAT2* at 8p21.3-23.1 and 8p22) and are inherited co-dominantly. The two enzymes are 87% homologous at the nucleotide level [13,14]. However, both genes are highly polymorphic among populations. A consensus nomenclature relates the genetic polymorphisms of NAT1 and NAT2 with respective acetylation phenotypes, which can be slow, fast or intermediate (http://nat.mbg.duth.gr/). Studies of allelic variants identified the NAT2 slow acetylation phenotype and the NAT1 rapid acetylation phenotype as possible factors in the development of a variety of tumors or ALL in children [9,12,15–17].

Regarding *NAT1* and *NAT2* SNPs and their relationship with ALL, different frequencies and associations with haplotypes between populations have been reported [3,9]. In a French–Canadian population, the haplotypes *NAT1*4* (reference), *NAT2*5C* (341T>C, 803A>G) and *NAT2*7B* (282C>T, 857G>A) showed odds ratios (ORs) of 1.9, 3.1 and 2.9, respectively [9]; for a Brazilian population, ORs of 3.8 for *NAT2*14A* (191G>A), 2.4 for *NAT2*5A* (341T>C, 481C>T) and 3.3 for *NAT2*5C* (341T>C, 803A>G) [12] have been reported. In an Egyptian population, ORs of 2.7 and 3.5 were reported for the haplotypes *NAT2*7A* (857G>A) and *NAT2*7B* (282C>T, 857 G>A),

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Supplemental data for this article can be accessed here.

respectively. Genetic studies of these NATs in ALL show a great genetic variability by population. In Mexico, there are no genotypic studies of polymorphisms of *NAT1* and *NAT2* in pediatric populations and only a few results for the allelic distribution of *NAT2* in healthy adult tuberculosis patients in isoniazid therapy or in patients with various types of cancer [18–21].

Therefore, the objective of this study was to perform genetic analysis of the main alleles encoding *NAT1* and *NAT2* enzymes in Mexican pediatric patients with ALL and to determine their possible association with the likelihood of developing this type of cancer.

Materials and methods

Subjects

In the present study, 57 unrelated pediatric patients aged 1 to 17 years with confirmed diagnosis of ALL were recruited from the Hospital Central "Dr. Ignacio Morones Prieto" (HCIMP) of San Luis Potosí, Mexico. Parents gave written informed consent. In addition, for patients aged 12 to 17 years, informed assent was obtained. Flow cytometry analysis was performed to determine the affected cell line in each patient with ALL. Several monoclonal antibodies against CD10, CD19, CD20, CD22, CD34, CD79a, TdT, IgMs and IgMc were used. All patients were classified as subtype B and received oncological treatment described in the Mexican clinical guidelines with drugs such as mercaptopurine, methotrexate, and L-asparaginase [22]. Patients included were assigned to a risk group based on predictive factors established in the literature [22–24]: 50 of 57 were at high risk, and 7 of 57 were at low risk. Although 87% of the patients were at high risk, only 12% had died by the 3-year follow-up. The control group, considered as a reference population, included 225 unrelated healthy volunteers (18–65 years old) who met the inclusion criteria, with three generations of ancestors born in the state of San Luis Potosí and who provided written informed consent. It is important to note that the controls were not matched for age because the genetic makeup of an individual does not change with time, and being adults guarantees that they did not and will not develop pediatric ALL, as previous studies have reported [3,9,11]. The protocol was approved by the HCIMP Ethics Committee (number 56-15).

DNA isolation

Genomic DNA was isolated from peripheral blood and extracted using a commercial Wizard® Genomic DNA Purification Kit (Promega Corporation, Madison, WI). DNA was quantified spectrophotometrically with a UV/VIS Optizen Pop® (Mecasys, Daejeon, Korea) and stored at −20 °C until the experiments were performed.

Genotyping

Analyses of the *NAT1* and *NAT2* alleles were performed as described in a previous assay [3,9,12]. Real-time polymerase chain reaction analysis was performed with the use of corresponding reagents (primers, Taq polymerase, dNTPs, magnesium chloride, TaqMan® fluorogenic probes) (Applied Biosystems®, Foster City, CA) using a previously standardized technique [21]. For the *NAT1* gene, four SNPs were analyzed: rs5030839 (559C>T), rs4986782 (560G>A), rs1057126 (1088T>A) and rs15561 (1095C>A); for the *NAT2* gene, six SNPs were analyzed: rs1041983 (282C>T), rs1801280 (341T>C), rs1799929 (481C>T), rs1799930 (590G>A), rs1208 (803A>G) and rs1799931 (857G>A). Once the presence of SNPs was determined, NAT haplotypes were assigned according to the consensus nomenclature (http://nat.mbg.duth.gr/).

Statistical analysis

To determine the allelic and genotypic frequencies and to test for Hardy–Weinberg equilibrium (HWE), genotyping data from both groups were analyzed using GenAlex® V6.501 (Australian National University) and Genetix® V 4.05.2 software (http://kimura.univ-montp2.fr/genetix/). A Bonferroni correction test was performed, in which $p > (.05/n$ loci) indicated that the population was in HWE. For linkage disequilibrium (LD) analysis, the software programs Arlequin® V3.1 (http://cmpg.unibe.ch/software/arlequin3/) and Fstat® V2.9.3.2 (Lausanne, Switzerland) were used to determine LD or linkage equilibrium (LE), and the values of P and D' were used. The level of significance was calculated by Fisher's exact test ($p < .05$). ORs were used to measure the strength of the association between the tested haplotypes, diplotypes and phenotypes with the likelihood of developing ALL. Crude ORs are given with the corresponding 95% confidence intervals (95%CIs). All statistical tests were based on a two-tailed probability and were performed using GraphPad Prism V 5.01 (GraphPad Software Inc., La Jolla, CA).

Results

According to independent analyses, the *NAT1* ($p \leq .000083$; D', 1) and *NAT2* ($p \leq .000033$, D', 1) loci were in LD; however, when both genes were analyzed,

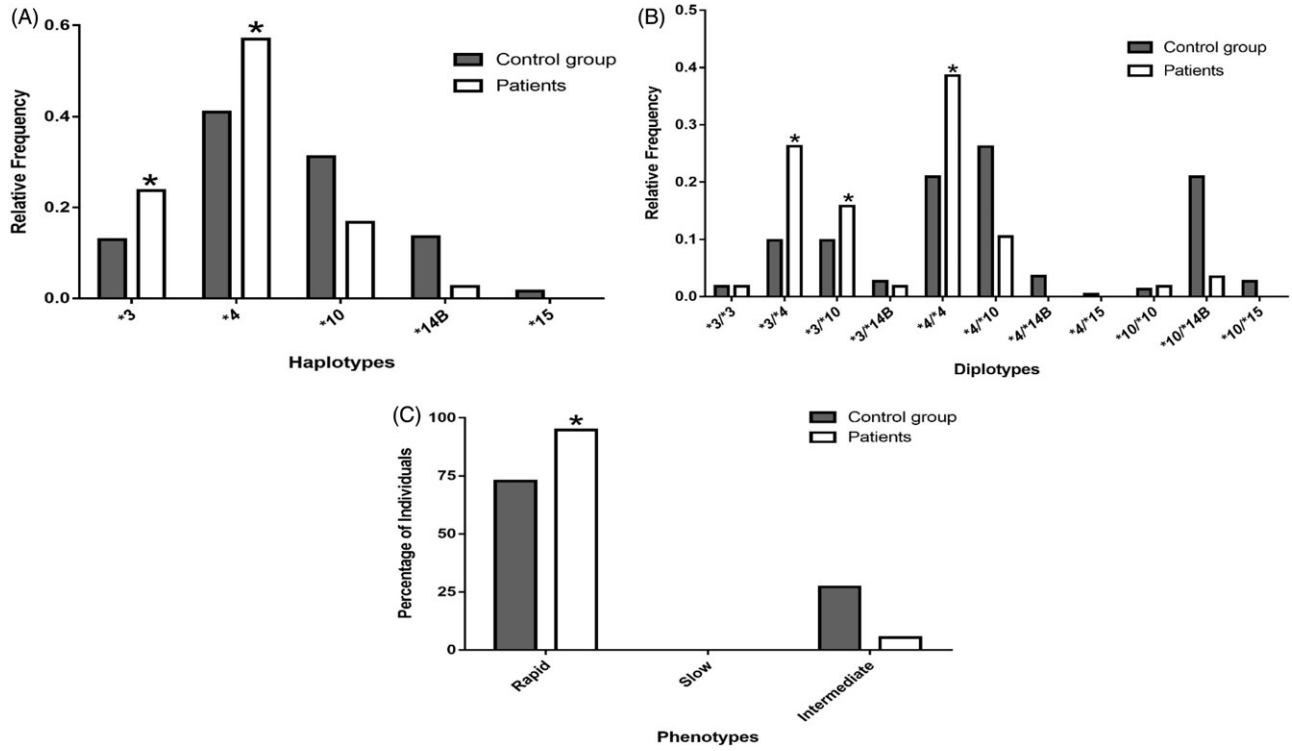

Figure 1. Distribution of haplotypes, diplotypes and phenotypes of NAT1. Comparison of the relative frequencies of haplotypes of NAT1 in the ALL and control groups (A). Comparison of the relative frequencies of diplotypes of NAT1 in the ALL and control groups (B). Comparison of phenotypes of NAT1 in percentages in ALL and control group. The slow phenotype was not identified in this study, and the rapid phenotype was present in a greater proportion in patients (C). Fisher's exact test was employed for the analysis. ALL: acute lymphoblastic leukemia; NAT: arylamine N-acetyltransferases.

Table 1. Frequency of the *NAT1* haplotype in patients with ALL and controls.

		Patients 57 (114)		Control group 225 (450)				
Haplotype	Phenotype	*n*	%	*n*	%	OR[a]	95% CI	*p* Value
*NAT1*4*	Rapid	65	57	184	40.89	**1.92**	**1.26–2.9**	**.0022**
*NAT1*3*	Rapid	27	23.68	58	12.89	**2.10**	**1.25–3.5**	**.0078**
*NAT1*10*	Rapid	19	16.66	140	31.11	0.44	0.26–0.75	.0023
*NAT1*14B*	Slow	3	2.63	61	13.56	0.17	0.05–0.55	.0004
*NAT1*15*	Truncated protein/no enzyme activity	0	0	7	1.56	DA[b]	DA	DA

[a]OR was calculated from the ratio of the number of haplotypes of interest *versus* all of the other haplotypes in patients compared with the ratio in control individuals.
[b]DA: Does not apply.
*NAT1*3* and *NAT1*4* showed a statistical association with the probability of developing ALL. (OR > 1 and $p < .05$).
ALL: acute lymphoblastic leukemia; NAT: arylamine N-acetyltransferases; ORs: odds ratio; CI: confidence intervals.

not all showed LD, LE or a tendency to equilibrium (Supplementary Tables 1, 2 and 3). *NAT1* was not found in HWE in either the control group (F_{is}, 0.21; $p < .0001$) or the ALL patients group (F_{is}, 0.29; $p < .0001$). In contrast, *NAT2* was found in HWE in both groups (F_{is} control group –0.12; $p = 1$; F_{is} ALL patient group –0.22; $p = 1$; Supplementary Tables 4 and 5).

Figure 1(A) shows that the haplotypes *NAT1*3* (1095C>A) and *NAT1*4* (reference) had higher relative frequencies in ALL patients than in the control group; however, the results differed for the haplotype *NAT1*10* (1088T>A, 1095C>A). This result indicates a possible statistical association between the likelihood of developing ALL and the first two haplotypes (*NAT1*3* and *NAT1*4*); however, the haplotype *NAT1*10,* as well as the other two mentioned above, are linked to the rapid phenotype (Table 1).

Diplotypes of *NAT1*3/*4, NAT1*3/*10* and *NAT1*4/*4* (rapid phenotype) showed significant associations with the likelihood of developing ALL (Figure 1(B); Table 2). These last results allowed us to determine the distribution of the NAT1 phenotype. A higher percentage of the rapid acetylator phenotype was observed in ALL patients, unlike in the control

Table 2. Frequency of the *NAT1* diplotype and phenotype in patients with ALL and controls.

		Patients 57		Control group 225				
Diplotype	Phenotype	*n*	%	*n*	%	OR	95% CI	*p* Value
*NAT1*3/*4*	Rapid	15	26.32	22	9.78	**3.29**	**1.57–6.8**	**.0033**
*NAT1*3/*10*	Rapid	9	15.78	22	9.77	1.73	0.75–4.0	.234
*NAT1*4/*4*	Rapid	22	38.60	47	20.89	**2.38**	**1.28–4.44**	**.009**
NAT1 *10/*14B	Intermediate	2	3.51	47	20.89	0.14	0.03–0.58	.0013
	Total of NAT1 rapid	54	94.7	164	72.8	**6.69**	**2–22**	**.0002**

The diplotypes described were the most frequent in this study. *NAT1*3/*4* and *NAT1*4/*4* showed a statistical association with the probability of developing ALL. The total number of the rapid phenotype of NAT1 was determined with all rapid acetylators classified by diplotype. (OR > 1 and $p < .05$).
ALL: acute lymphoblastic leukemia; NAT: arylamine N-acetyltransferases; ORs: odds ratio; CI: confidence intervals.

group (Figure 1(C)). The rapid phenotype presented an OR of 6.69, indicating the likelihood of developing ALL compared with the intermediate phenotype (95%CI, 2–22; $p = .0002$; Table 2). A significant association was found between the likelihood of developing ALL and the presence of the haplotypes *NAT1*3* or *NAT1*4*; an OR of 2.1 (95%CI, 1.25–3.5; $p = .0078$) was obtained for the first haplotype, and an OR of 1.92 (95%CI, 1.26–2.9; $p = .0022$) was obtained for the second. In contrast, no statistical association with the haplotype *NAT1*10* (OR = 0.44) (95%CI, 0.26–0.75; $p = .0023$) was detected (Table 1).

The diplotypes *NAT1*3/*4* and *NAT1*4/*4* presented statistically significant results (Table 2), the first with an OR = 3.29 (95%CI, 1.57–6.8; $p = .0033$) and the second with an OR = 2.38 (95%CI, 1.28–4.44; $p = .009$). For diplotypes that presented the haplotype *NAT1*10,* no association was observed. Accordingly, when an analysis of rapid phenotypes lacking the *NAT1*10* haplotype was performed, an OR = 4 (95%CI, 2–7.5; $p < .0001$) was obtained, indicating that this haplotype was not significantly associated with the phenotype. A similar analysis was performed for the rapid diplotypes with at least one haplotype, *NAT1*3 (*X/*3)* (OR = 2.8) (95%CI, 1.5–5.3; $p = .0011$) or *NAT1*4 (*X/*4)* (OR = 2.2) (95%CI, 1.1–4.4; $p = .0147$). "X" represents any haplotype of the subject but related to the second haplotype of interest. In the case of **X/*4,* a third analysis without *NAT1*4/*4* was conducted, obtaining an OR = 1.0 (95%CI, 0.55–1.8; $p = 1$), which assigns statistical significance to the presence of this diplotype.

Haplotype and diplotype analysis of the *NAT2* gene showed a wide variability (Figure 2). The relative frequencies of the haplotypes *NAT2*6B* (590G>A), *NAT2*6J* (282C>T, 590G>A, 857G>A) and *NAT2*7A* (857G>A), and the diplotypes *NAT2*5B/*6B, 5*B/*6J, *6A/*7A, *6B/*7A, *6J/*7A* and *6J/*7B* were higher in ALL patients than in the control group. The three NAT2 acetylator phenotypes (rapid, intermediate and slow) were identified in the control group, whereas in ALL patients, only two types were observed, with a high percentage of slow acetylators (Figure 2(C)).

Tables 3 and 4 show the association analysis for the likelihood of developing ALL with the slow phenotype (OR, 2.95; 95%CI, 1.47–5.87; $p = .0014$), as well as the haplotypes *NAT2*6B* (OR, 3.3; 95%CI, 1.8–5.9; $p < .0001$), *NAT2*6J* (OR, 3.25; 95%CI, 1.67–6.32; $p = .0008$) and *NAT2*7A* (OR, 2.45; 95%CI, 1.3–4.62; $p = .0074$).

Due to the wide variability of diplotypes of *NAT2*, those with higher frequencies were analyzed in ALL patients, including *NAT2*5B/*6B* (OR = 3.3, 95%CI, 1.4–7.7; $p = .008$), *NAT2*5B/*6J* (OR = 2.1, 95%CI, 0.86–5.2; $p = .11$), *NAT2*6B/*7A* (OR = 16.9, 95%CI, 1.8–154; $p = .0065$), *NAT2*6J/*7A* (OR = 4.2, 95%CI, 1.1–15.1; $p = .016$) and *NAT2*6J/*7B* (OR = 8.4, 95%CI, 1.5–47; $p = .01$).

In addition, due to the large 95%CI of each diplotype, association by family was analyzed with the aforementioned diplotypes (Table 4). The NAT2 slow phenotype was determined based on the combinations of the haplotypes *5, *6 and *7, identified in this study.

Discussion

NAT enzymes have been extensively studied because of their important role in the metabolism of aromatic arylamines and the presence of SNPs that influence their acetylation activity. Previously, we studied *NAT1* (unpublished data) and *NAT2* [21] in healthy adults and in tuberculosis patients treated with isoniazid, demonstrating that both enzymes have high genetic variability in our population.

Both genes were found to be in LD because they share the same chromosomal location and present 87% nucleotide sequence homology [14,25,26]. However, the SNPs of the *NAT1* gene found in the population studied were not in HWE. Consequently, the NAT1 results are only relevant for the current population. Generally, it is common for genetic association studies not to provide this information [13]; therefore, this study is among the first to report the HWE findings of NATs in ALL. In contrast, *NAT2* SNPs were in HWE in both groups, consistent with several other studies [3,12,26,27]. In this context, *NAT2* SNPs

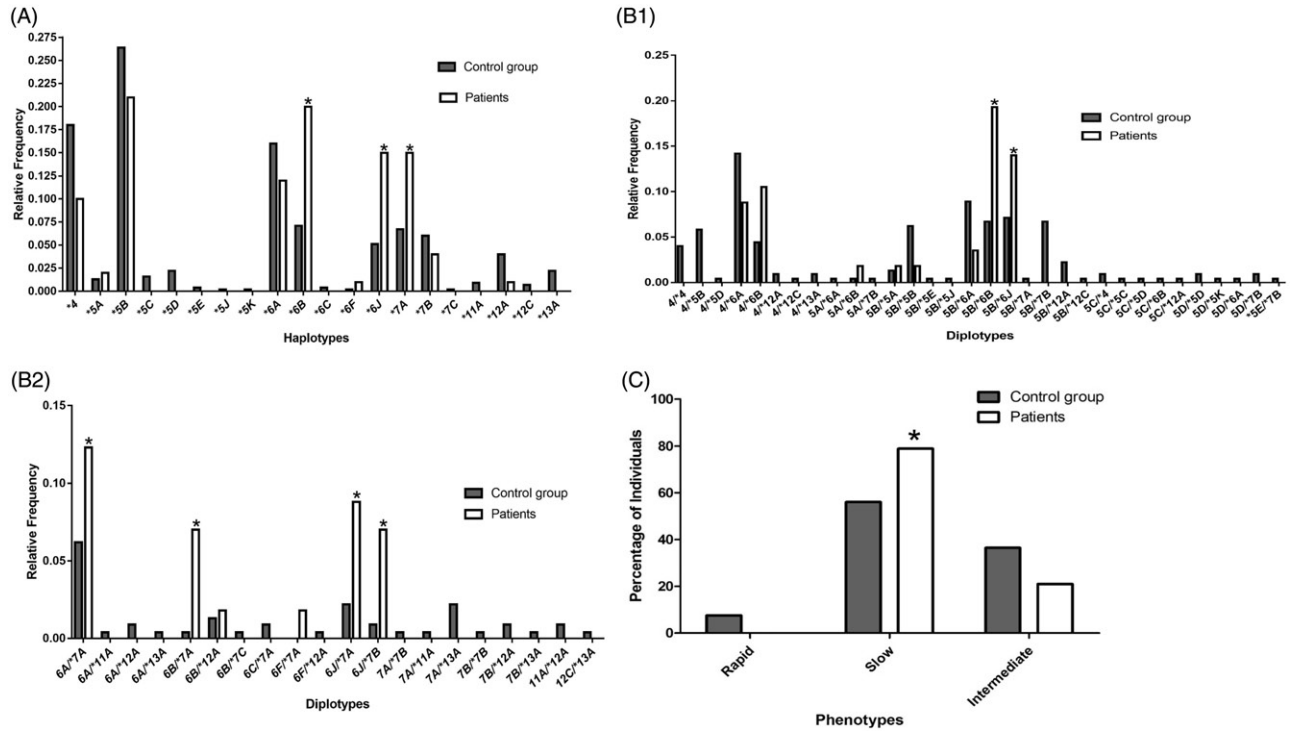

Figure 2. Distribution of haplotypes, diplotypes and phenotypes of NAT2. Comparison of the relative frequencies of haplotypes of NAT2 in the ALL and control groups (A). Comparison of the relative frequencies of diplotypes of NAT2 in the ALL and control groups (52 diplotypes were identified) (B). Comparison of phenotypes of NAT2 in percentages in the ALL and control groups. The rapid phenotype was not identified in patients, and the slow phenotype was present in a greater proportion in patients (C). Fisher's exact test was employed for the analysis. ALL: acute lymphoblastic leukemia; NAT: arylamine N-acetyltransferases.

Table 3. Frequency of the *NAT2* haplotype in patients with ALL and controls.

		Patients 57 (114)		Control group 225 (450)				
Haplotype	Phenotype	*n*	%	*n*	%	OR	95% CI	*p* Value
*NAT2*4*	Rapid	11	9.65	81	18.00	0.48	0.25–0.95	.03
*NAT2*5B*	Slow	24	21.05	119	26.44	0.74	0.45–1.22	.2783
*NAT2*6A*	Slow	14	12.28	72	16.00	0.73	0.39–1.35	.38
*NAT2*6B*	Slow	23	20.18	32	7.11	**3.30**	**1.84–5.90**	**.0001**
*NAT2*6J*	Slow	17	14.91	23	5.11	**3.25**	**1.67–6.32**	**.0008**
*NAT2*7A*	Slow	17	14.91	30	6.67	**2.45**	**1.30–4.62**	**.0074**
*NAT2*7B*	Slow	4	3.51	27	6.00	0.57	0.19–1.66	.36

The haplotypes described were the most frequent in this study, *NAT2*6B, NAT2*6J* and *NAT2*7A* showing a statistical association with the probability of developing ALL. (OR > 1 and $p < .05$).
ALL: acute lymphoblastic leukemia; NAT: arylamine N-acetyltransferases; ORs: odds ratio; CI: confidence intervals.

Table 4. Frequency of *NAT1* diplotype and phenotype in patients with ALL and controls.

	Patients 57		Control group 225				
Diplotype/ phenotype	*n*	%	*n*	%	OR	95% CI	*p* Value
*NAT2*5/*6*	22	38.6	55	24.44	**1.94**	**1.05–3.59**	**.044**
*NAT2*6/*7*	21	36.8	25	11.11	**4.667**	**2.36–9.2**	**<.0001**
Total of NAT2 Slow	45	78.90	126	56.00	**2.95**	**1.47–5.87**	**.0014**

OR was calculated from all diplotypes detected. However, the confidence intervals obtained were very broad, so OR was calculated by diplotype family (slow acetylators). The total number of the rapid phenotype of NAT2 was determined with all slow acetylators classified by diplotype. (OR > 1 and $p < .05$).
ALL: acute lymphoblastic leukemia; NAT: arylamine N-acetyltransferases; ORs: odds ratio; CI: confidence intervals.

are often present in HWE in different ethnic groups, unlike those of *NAT1*.

A statistical association between the probability of developing ALL in the rapid phenotype of NAT1 was found. In this case, it is possible that subjects who rapidly acetylate xenobiotic compounds (specifically toxic compounds) to highly reactive products are at an increased risk of mutations with the consequent alteration of the cell cycle [14,26,28–30]. In this study, *NAT1*3* and *NAT1*4* haplotypes were associated with ALL. In contrast, *NAT1*10,* which has been statistically associated with other types of cancer (colon, lung, breast, prostate), was not associated with ALL [31–35]. Krajinovic et al. [9] reported a statistical association between *NAT1*4* and ALL. It is of interest to study the effects of these NAT1 haplotypes on its enzymatic activity and the acetylation of xenobiotic reactions. It has been reported that the NAT1 enzyme may have higher affinities for O- and N,O- acetylation reactions [14,31,36]; however, the effect of each transformed molecule in cancer development has not been described [29].

The increased likelihood of developing ALL in the presence of the *NAT1*3/*4* and *NAT1*4/*4* diplotypes but not *NAT1*3/*3* or any diplotype, including *NAT1*10*, indicates that *NAT1*10* may provide a protective effect against ALL. In contrast, for NAT2, an association between the slow phenotype and the likelihood of developing ALL was found [3,9,12].

The NAT2 enzyme, whose function is to participate in N-acetylation reactions, produces less reactive, more polar metabolites that are quickly removed from the body [31]. Families of the haplotypes *NAT2*5, NAT2*6* and *NAT2*7* are characterized by the slow phenotype (http://nat.mbg.duth.gr/). In this study, a statistical relationship was found between the likelihood of developing ALL and the haplotypes of *NAT2*6* and *NAT2*7* families, specifically with *NAT2*6B, NAT2*6J* and *NAT2*7A*. This relationship was not demonstrated for haplotypes of the family *NAT2*5*, particularly *NAT2*5B,* despite its prevalence in the control group.

Selinski et al. [37] have proposed a new classification for the phenotypes of NAT2, in which the haplotypes *NAT2*6* and *NAT2*7* are considered "ultra-slow acetylators" with respect to *NAT2*5*. This classification would explain why, in our studied group, greater statistical power was observed in the diplotype of the *NAT2*6/*7* family (Table 4). Other reports have described the great genetic variability of NAT enzymes in populations in Brazil [12] and Africa [28] (Supplementary Tables 6 and 7), but this study is the first to report the haplotypes of the NAT enzyme genes in a Mexican pediatric population as indicative of their high heterogeneity.

Of the 57 patients, 20 were in the maintenance phase, 28 were in surveillance, 1 was in relapse, 1 did not respond to the treatment and 7 unfortunately died. Of these last seven patients, six were diagnosed as high risk, and one was low risk. All patients had at least one of the risk haplotypes identified in this study; however, the risk haplotypes were also present in patients in the surveillance phase, whereby risk haplotypes have no influence on disease prognosis.

Based on the results of this study and due to the complex metabolism of xenobiotics, it is important to conduct additional genetic association studies on ALL with other enzymes involved in the metabolism of polycyclic aromatic hydrocarbons and aromatic and heterocyclic amines, such as GSTM1, CYP2E1, CYP1A1 and MTRR, for which it has already been reported that there are significant statistical associations [8,9,11]. In addition, it is important to conduct more research on the toxicology and molecular biology of toxic compounds that predominate in places where patients live to elucidate how these enzymes act in the process of the detoxification of these compounds and to define what types of metabolites are generated and how they interact at the cellular level.

Conclusions

The results of this first genetic association study in Mexican pediatric patients with ALL demonstrate the NAT1 rapid haplotypes *NAT1*4* and *NAT1*3*, as well as the NAT2 slow haplotypes *NAT2*6B, NAT2*6J* and *NAT2*7A,* are associated with the likelihood of developing ALL. This study provides the basis for further research on the genetic factors that promote the development of this type of cancer.

Potential conflict of interest: Disclosure forms provided by the authors are available with the full text of this article online at https://doi.org/10.1080/10428194.2017.1406090

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