Universidade Estadual de Londrina

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ANÁLISE DE POLIMORFISMOS GENÉTICOS DAS GLUTATIONA-STRANSFERASES GSTM1 E GSTT1 E NAD (P) H QUINONA OXIDOREDUTASE (NQO1) NA LEUCEMIA LINFOIDE AGUDA INFANTOJUVENIL:

SUSCETIBILIDADE E PROGNÓSTICO

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Tese apresentada ao Programa de Pós-Graduação em Patologia Experimental da Universidade Estadual de Londrina como requisito para obtenção do título de Doutor.

Orientador: Prof.ª Drª. Maria Angelica Ehara Watanabe.
Coorientador: Prof. Dr. Carlos Eduardo Coral de Oliveira.

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Londrina, 16 de dezembro de 2019.

FUJITA, T. C. **Análise de polimorfismos genéticos das glutationa-stransferases GSTM1 e GSTT1 e NAD (P) H quinona oxidoredutase (NQO1) na leucemia linfoide aguda infantojuvenil:** suscetibilidade e prognóstico. 2019. 81 p. Tese (Doutorado em Patologia Experimental) – Universidade Estadual de Londrina, Londrina, 2019.

RESUMO

A leucemia é a forma mais frequente de câncer em crianças e adolescentes com menos de 14 anos de idade, representando cerca de 31% das doenças malignas antes dos 15 anos de idade. A leucemia linfoblástica aguda (LLA) é uma neoplasia hematológica caracterizada por um acúmulo excessivo de células progenitoras imaturas linfoides no sangue e na medula óssea. Trata-se de doença heterogênea em relação as alterações genéticas, resposta ao tratamento e prognóstico. Embora a sobrevida global dos pacientes com LLA na infância após a quimioterapia tenha melhorado ao longo dos anos, atingindo taxas de cura de 80%, muitos estudos estão sendo desenvolvidos na tentativa de melhorar o diagnóstico e a eficácia dos protocolos terapêuticos atuais. As glutationas S-transferases (GSTs), como como GSTM1 e GSTT1 e a NAD (P) H quinona oxidoredutase 1 (NQO1) são enzimas envolvidas na fase II das reações de detoxificação. As quinonas são uma classe de compostos orgânicos derivados de hidrocarbonetos aromáticos, encontrados em vários sistemas biológicos. Envolvida no metabolismo das quinonas, a enzima NQO1 é uma redutase que protege contra os efeitos tóxicos dos medicamentos anticâncer. O gene NQO1 possui um polimorfismo rs1800566 (C609T), o que leva a uma baixa atividade enzimática. Como a enzima codificada por esse gene realiza uma etapa importante na homeostase celular, fica claro que a ausência ou diminuição de suas atividades podem levar a um aumento na toxicidade e suscetibilidade ao desenvolvimento de neoplasias malignas, como a LLA. Os genes GSTM1 e GSTT1 têm um polimorfismo que causa a exclusão homozigótica do gene e leva a atividade enzimática alterada. Assim, o presente estudo avaliou os polimorfismos nos genes NQO1, GSTT1 e GSTM1 em pacientes com LLA e controles livres de neoplasia e analisou os níveis plasmáticos de GST no risco de progressão e resposta ao tratamento. Amostras de sangue periférico de 74 pacientes com diagnóstico confirmado e 115 controles tiveram seus DNAs genômicos extraídos e amplificados por metodologia baseada na reação em cadeia da polimerase (PCR) para o polimorfismo do NQO1. O estudo caso-controle não indicou associação entre a presença das variantes polimórficas e a suscetibilidade a LLA ou sobrevida (OR: 0.83, CI95%: 0.50-1.39). No entanto, este polimorfismo foi correlacionado com a recidiva na LLA (p = 0,03). No outro estudo, 64 pacientes com LLA e 68 controles foram analisados quanto aos polimorfismos dos genes GSTT1 e GSTM1 e os níveis plasmáticos de GST. Ambos os polimorfismos não foram associados à suscetibilidade (GSTM1.OR: 0.70, CI95%: 0.35 - 1.43; GSTT1. OR: 1.12, CI95%: 0.47-2.65) ou risco de recidiva de LLA e seus diferentes genótipos não alteraram os níveis plasmáticos de GST. Entretanto, verificou-se aumento da concentração de GST na LLA em relação ao grupo controle (p<0,0001), mas não foi encontrada associação dos níveis plasmáticos de GST com os parâmetros analisados. Assim, nas amostras analisadas, o gene NQO1 foi apontado como um possível candidato a marcador molecular de prognóstico ou progressão para a oncogênese da LLA, e os

níveis aumentados de GST no plasma de pacientes com LLA sugerem um possível envolvimento no tratamento.

Palavras-chave: prognóstico; recidiva; polimorfismo genético.

FUJITA, T. C. **Analysis of genetic polymorphisms of glutationa-stransferases GSTM1 and GSTT1 and NAD (P) H quinona oxidoredutase (NQO1) in children's acute lymphoside leukemia:** susgenicity and prognosis. 2019. 81 f. Thesis (Doctorate in Experimental Pathology) – Londrina State University, Londrina, 2019.

ABSTRACT

Leukemia is the most common form of cancer in children and adolescents under 14 years of age, representing about 31% of malignant diseases before 15 years of age. Acute lymphoblastic leukemia (ALL) is a hematological neoplasia characterized by an excessive accumulation of immature lymphoid progenitor cells in the blood and bone marrow. It is a heterogeneous disease in relation to genetic changes, response to treatment and prognosis. Although the overall survival of ALL patients in childhood after chemotherapy has improved over the years, reaching cure rates of 80%, many studies are being developed in an attempt to improve the diagnosis and the effectiveness of current therapeutic protocols. Glutathione S-transferases (GSTs), such as GSTM1 and GSTT1 and NAD (P) H quinone oxidoreductase 1 (NQO1) are enzymes involved in phase II of detoxification reactions. Quinones are a class of organic compounds derived from aromatic hydrocarbons, found in various biological systems. Involved in the metabolism of quinones, the enzyme NQO1 is a reductase that protects against the toxic effects of anticâncer drugs. The NQO1 gene has a rs1800566 (C609T) polymorphism, which leads to low enzyme activity. As the enzyme encoded by this gene performs an important step in cell homeostasis, it is clear that the absence or decrease in its activities can lead to an increase in toxicity and susceptibility to the development of malignant neoplasms, such as ALL. The GSTM1 and GSTT1 genes have a polymorphism that causes homozygous gene exclusion and leads to altered enzyme activity. Thus, the present study evaluated the polymorphisms in the NQO1, GSTT1 and GSTM1 genes in patients with ALL and neoplasia-free controls and analyzed the plasma levels of GST in the risk of progression and response to treatment. Peripheral blood samples from 74 patients with confirmed diagnosis and 115 controls had their genomic DNA extracted and amplified by a methodology based on the polymerase chain reaction (PCR) for the NQO1 polymorphism. The case-control study did not indicate an association between the presence of polymorphic variants and susceptibility to ALL or survival (OR: 0.83, CI95%: 0.50-1.39). However, this polymorphism was correlated with relapse in ALL ($p = 0.03$). In the other study, 64 ALL patients and 68 controls were analyzed for polymorphisms of the GSTT1 and GSTM1 genes and plasma GST levels. Both polymorphisms were not associated with susceptibility (GSTM1.OR: 0.70, CI95%: 0.35 - 1.43; GSTT1. OR: 1.12, CI95%: 0.47-2.65) or risk of ALL recurrence and their different genotypes did not change plasma levels of GST. However, there was an increase in the concentration of GST in the ALL compared to the control group ($p <0.0001$), but no association was found between plasma GST levels and the parameters analyzed. Thus, in the samples analyzed, the NQO1 gene was identified as a possible candidate for a molecular marker of prognosis or progression to ALL oncogenesis, and the increased levels of GST in the plasma of patients with ALL suggest a possible involvement in the treatment.

Keywords: prognosis; relapse; genetic polymorphism.

LISTA DE TABELAS

LISTA DE ABREVIATURAS E SIGLAS

A	Adenina
ACS	Sociedade Americana de Câncer (*American Cancer Society*)
$AgNO_3$	Nitrato de prata
AML1	Gene da leucemia mieloide aguda 1 (conhecido como *RUNX1*)
AMP_c	3',5' Monofosfato de adenosina cíclico
APEX1	Gene da endodeoxirribonuclease 1 de apurínica e apirimidínica (*apurinic/apyrimidinic endodeoxyribonuclease 1 gene*)
ATM	Gene da ataxia telangiectasia mutada
BMP	Proteína Morfogenética Óssea
bp	Par de base
BRCA2	Gene da proteína de susceptibilidade ao câncer de mama do tipo 2 (*Breast Cancer Type 2 susceptibility protein*)
C	Citosina
CALLA	Antígeno comum da leucemia linfoide aguda
CD	Marcadores ou grupo de diferenciação
CDK6	Gene quinase 6 dependente de ciclina
CDKN2A	Gene do inibidor 2A de quinases dependentes de ciclinas
CEP	Comitê de Ética em Pesquisa em Seres Humanos
C-MYC	Gene da mielocitomatose celular
CREB	Proteína ligante ao elemento de resposta ao AMP_c
del	Deleção
DNA	Ácido desoxirribonucleico
dNTP	Desoxirribonucleotídeo trifosfatado
E2A	Fator de transcrição intensificador de ligação do gene E12 / gene *E47* da Ig
EBV	Vírus *Epstein Barr*
EDTA	Ácido etilenodiamino tetra-acético
EGIL	Grupo Europeu de Classificação Imunológica das Leucemias
ELISA	Ensaio de imunoadsorção ligado à enzima (*Enzyme-Linked Immunosorbent Assay)*
ERCC2	Gene do grupo complementar da deficiência no reparo de complementação cruzada no reparo de excisão de roedores (*excision*

	repair cross-complementing rodent repair deficiency, complementation group 2 gene)
ETP	Precursor da célula T precoce
ETS	Gene do fator de transformação específica do eritroblasto (*Erythroblast transformation-specific*)
ETV6	Gene da variante 6 do fator de transcrição ETS
FAB	Grupo Franco-Américo-Britânico
FISH	Hibridização fluorescente *in situ*
G	Guanina
GBTLI	Grupo Brasileiro de Tratamento de Leucemia na Infância
GDF	Fatores de crescimento e diferenciação
GDNF	Fatores neurotróficos derivados da glia
H_2O_2	Peróxido de hidrogênio
H_3PO_4	Ácido fosfórico
HCL	Hospital do Câncer de Londrina
HLA-DR	Antígeno Leucocitário Humano – classe II DR
HLF	Gene do fator hepático da leucemia
HOX	Gene *Homeobox*
HSC	Células-tronco hematopoiéticas
HTLV-1	Vírus Linfotrópico da célula T humana 1
iAMP21	Gene da amplificação intracromossomal do cromossomo 21
IC	Intervalo de Confiança
Ig	Imunoglobulina
IGH	Gene da cadeia pesada da imunoglobulina
IL-10	Interleucina 10
IL3	Gene da interleucina 3
INCA	Instituto Nacional de Câncer
KLR	Gene do receptor do tipo lectina do membro 1 da subfamília K
KMT2A	Gene da lisina metiltransferase 2A
LDH	Lactato desidrogenase
LLA	Leucemia Linfoide Aguda
LMA	Leucemia Mieloide Aguda
LNH	Linfoma não-Hodgkin
$MgCl_2$	Cloreto de magnésio

miR	Micro RNA pequeno não-codificador
MLL	Gene da leucemia de linhagem mista ou linfoide/mieloide
MO	Medula óssea
MPO	Enzima mieloperoxidase
MRD	Doença Residual Mínima
mRNA	RNA mensageiro
NaOH	Hidróxido de sódio
NK	Células *Natural Killer*
NKG2D	Gene da célula NK do membro D do grupo 2
Notch1	Gene da proteína homóloga 1 a Notch loco neurogênico
OMS	Organização Mundial de Saúde
OR	Razão de chances (*Odds Ratio*)
PAS	Ácido periódico de Schiff
PCR	Reação em cadeia da polimerase
PBX1	Gene *homeobox* 1 da leucemia de célula pré-B
RAD51	Gene da RAD51 recombinase da *Saccaromyces cerevisiae*
Rb	Proteína retinoblastoma
RCBP	Registros de Câncer de Base Populacional
RFLP	Polimorfismo de Comprimentos de Fragmentos de Restrição
RNA	Ácido ribonucleico
RT-PCR	Reação em cadeia da polimerase por transcriptase reversa
RUNX1	Gene do fator de transcrição relacionada ao runt-1
SNC	Sistema nervoso central
SNP	Polimorfismo de base única
t	Translocação
T	Timina
TAL	Gene da leucemia linfoblástica aguda de célula T
Taq	*Thermus aquaticus*
TCF3	Gene do fator de transcrição 3 (também conhecido como *E2A*)
TCR	Gene do receptor da célula T
TdT	Enzima nuclear deoxinucleotidil transferase terminal
TEL	Oncogene *TEL* (também conhecido como *ETV6*)
TGP	Transaminase glutâmico pirúvica
TGO	Transaminase glutâmico oxalacética

TMB	3,3', 5,5'-tetrametilbenzidina
XRCC4	Gene do complemento cruzado no reparo de raio-X (*X-ray repair cross complementing 4gene*)

SUMÁRIO

1 INTRODUÇÃO

No Brasil, o Instituto Nacional de Câncer (INCA) José Alencar Gomes da Silva estimou, para o biênio 2018-2019, 420 mil casos novos de câncer, excluindo tumores de pele não melanoma. Sendo que aproximadamente 12.500 casos acometeram crianças e adolescentes até os 19 anos. Levando em consideração as leucemias, foram estimados 5.940 novos casos em homens e 4.860 em mulheres, correspondendo a um risco estimado de 5,75 e 4,56 a cada 100 mil homens e mulheres, respectivamente (INCA, 2015).

O câncer infantojuvenil, que ocorre entre 0 e 19 anos, consiste em um conjunto de doenças que apresentam características próprias em relação as células que compõem os tumores, ou seja, o tipo histológico, e ao comportamento clínico da doença (Little, 1999). Na maioria das populações, o câncer infantojuvenil corresponde de 1% a 4% de todos os tumores malignos, sendo que nos países em desenvolvimento, onde a população de crianças chega a 50%, essa proporção do câncer infantil, representa de 3% a 10% do total de neoplasias. Já nos países desenvolvidos, essa proporção diminui, chegando a cerca de 1% (FERLAY, J. *et al.*, 2013; MAGRATH, I. *et al.*, 2013; SOCIETY, A. C., 2016). Entre os tipos de câncer infantojuvenis no mundo todo, a leucemia é o mais comum na maioria das populações, correspondendo de 25% a 35% dos casos (HOWLADER, N., NOONE, A. e KRAPCHO, M., 2014).

No Brasil, o percentual mediano de neoplasias nos Registros de Câncer de Base Populacional (RCBP) na população infantojuvenil foi de 3%. Assim como na maioria das populações, as leucemias foram as mais frequentes, com 26%. Em 2015, no país, ocorreram 2.704 óbitos por câncer infantojuvenil, o que corresponde a 7,9% entre todas as causas e a segunda maior causa de morte em todas regiões (INCA, 2017).

As leucemias são grupos de doenças hematológicas com conceito biológico, apresentação clínica, prognóstico e resposta ao tratamento diferentes, caracterizada pela presença de uma população de células anormais suprimindo a produção normal dos componentes celulares do sistema hematopoiético (POLYCHRONAKIS, I. *et al.*, 2013).

A origem das leucemias provém das células tronco hematopoiéticas (HSCs) e precursoras na medula óssea (MO), promovendo proliferação e infiltração das células leucêmicas (AZIZIDOOST, S. *et al.*, 2014; KONOPLEVA, M. Y. e JORDAN, C. T., 2011). De uma forma generalizada, as neoplasias hematológicas são classificadas de acordo com a linhagem, grau de maturação e forma de acometimento celular na MO. As neoplasias da linhagem mieloide podem incluir granulócitos (neutrófilos, eosinófilos, basófilos), monócitos,

eritrócitos, plaquetas (derivados dos megacariócitos) e mastócitos. Em contrapartida, da linhagem linfoide corresponde às leucemias de linfócitos B ou T e células *natural killer* (NK) (VARDIMAN, J. W. *et al.*, 2009).

1.1 LEUCEMIA LINFOIDE AGUDA

A leucemia linfoide aguda (LLA) é uma desordem maligna das células progenitoras da linhagem linfoide que afeta as faixas etárias da pediátrica à adulta (ARMSTRONG, S. A. e LOOK, A. T., 2005; PUI, C.-H., ROBISON, L. L. e LOOK, A. T., 2008), porém as crianças e adolescentes são os mais acometidos por esta neoplasia (ACS, 2016a).

Em 2016, de acordo com a Sociedade Americana de Câncer foram observados 6.590 novos casos de LLA em adultos, sendo 3.590 em homens e 3.000 em mulheres, e 1.430 mortes, sendo 800 homens e 630 mulheres (ACS, 2016b). No Brasil, a incidência de leucemia infantil, entre 1999 e 2010, registrou 74,52 casos por milhão de habitantes, com pico de incidência entre 4 e 5 anos em ambos os sexos, dos quais 67,77 dos casos registrados foram de LLA (DE SOUZA REIS, R. *et al.*, 2016).

Acredita-se que a LLA pode ser originada de interações entre exposições exógena e endógena, suscetibilidade genética (herdada) ou ao acaso (GREAVES, M. F. e WIEMELS, J., 2003; INABA, H., GREAVES, M. e MULLIGHAN, C. G., 2013).

Poucos fatores foram identificados como associados a um risco aumentado para LLA, dos quais pode-se incluir a exposição pré-natal a raios-x; a exposição pós-natal a altas doses de radiação; e condições genéticas bem específicas, como a Síndrome de Down, entre outras síndromes (INCA, 2015).

Além da exposição à radiação ionizante, outros fatores ambientais incluem os hidrocarbonetos, em especial o benzeno, os pesticidas, e o consumo de álcool, cigarro e drogas ilícitas durante a gestação, os quais têm sido descritos como fatores predisponentes à LLA infantil (HASHIBE, M. *et al.*, 2005; INFANTE-RIVARD, C. *et al.*, 1999; METAYER, C. *et al.*, 2013). Alguns agentes biológicos, como os vírus – vírus *Epstein Barr* (EBV) e vírus linfotrópico da célula T humana 1 (HTLV-1) – também permanecem como fatores de risco para LLA, no sentido de que podem desregular a resposta imunológica, ou ainda a ausência de contato com microorganismos durante a infância (GREAVES, M., 2006; INABA, H., GREAVES, M. e MULLIGHAN, C. G., 2013).

1.1.1 Classificação da LLA

A LLA se caracteriza pela proliferação de células imaturas derivadas da MO, chamada de blastos, que podem envolver o sangue periférico ou os órgãos sólidos, bem como o sistema nervoso central (SNC) e os testículos. A porcentagem de blastos necessários na MO para o diagnóstico das leucemias agudas foi ajustada para pelo menos 20%. Quando as características citogenéticas e morfológicas estão presentes, não há exigência de uma porcentagem mínima de blastos (ABDUL-HAMID, G., 2011).

Três modelos são utilizados para subclassificação da LLA: (1) modelo criado pelo grupo Franco-Américo-Britânico (FAB), que leva em consideração a análise morfológica e citoquímica (BENNETT, J. M. *et al.*, 1976); (2) modelo da Organização Mundial da Saúde (OMS) em 1976 e atualizada em 2016, que classifica pela citogenética (ARBER, D. A. *et al.*, 2016; MATHÉ, G. e RAPPAPORT, H., 1976) (3) e o Grupo Europeu de Classificação Imunológica das Leucemias (EGIL), que caracteriza pela imunofenotipagem (BENE, M. *et al.*, 1995).

A avaliação morfológica da MO representa o primeiro critério do diagnóstico na diferenciação da LLA e LMA, sendo útil para distinguir mieloblastos de linfoblastos (LAI, R., HIRSCH-GINSBERG, C. F. e BUESO-RAMOS, C., 2000). No entanto, vale ressaltar que a citometria de fluxo é considerada o padrão ouro para diagnóstico, com finalidade de identificar as diferentes linhagens celulares (CHIARETTI, S., ZINI, G. e BASSAN, R., 2014).

A ausência de um critério padronizado para classificação de subgrupos imunofenotípicos, de estudos prospectivos controlados no tratamento e de estratégias terapêuticas diferentes comprometeram a avaliação do impacto prognóstico dos estudos imunofenotípicos na LLA (LUDWIG, W. D., HAFERLACH, C. e SCHOCH, C., 2003). Para este problema o grupo EGIL propôs uma classificação para estratificar as leucemias agudas somente com base na imunofenotipagem pela citometria de fluxo (Tabela 1) (BENE, M. *et al.*, 1995), confirmando a quantificação citomorfológica de blastos e fornecendo informações sobre determinado subtipo e linhagem envolvidos (CRAIG, F. E. e FOON, K. A., 2008; MCKENNA, R. W., 2001).

Tabela 1. Classificação imunofenotípica das LLA segundo o EGIL.

Classificação	Imunofenótipo	Frequência	
LLA de linhagem B	HLADR, $CD19^+$, e/ou $CD79a^+$, e/ou $CD22^+$, e/ou $CD24^+$, e/ou $CD34^+$	Adultos	Crianças
BI (Pró-B)	$CD19^+$, $CD22^+$, $CD34^+$, TdT^+,$cCD79a^+$, $CD10^+$	11%	5-9%
BII (B comum)	$CD10^+$ (CALLA), TdT^+	49%	53-65%
BIII (Pré-B)	$CD19^+$, $CD22^+$, $CD34^+$,TdT^+, $cIgM^+$	12%	14-20%
BIV (B madura)	Ig (citoplasma ou superfície), TdT^-, $CD34^-$	2-4%	2-3%
LLA de linhagem T	CD1a, CD2, $CD3^+$ (citoplasma/superfície), CD4, CD5, CD7, CD8, CD34	25%	11-16%
TI (Pró-T)	$cCD3^+$, $CD7^+$	7%	
TII (Pré-T)	$cCD3^+$, $CD7^+$, $CD2^+$ e/ou $CD5^+$ e/ou $CD8^+$		
TIII (T cortical ou tímica)	$CD1a^+$, $cCD3^+$, $sCD3^{+/-}$	17%	
TIV (T madura)	$cCD3^+$, $sCD3^+$, $CD1a^-$	1%	

CD: marcadores de diferenciação *(cluster of differentiation*); c: citoplasmático; s: superfície; CALLA: antígeno comum da LLA; HLA-DR: Antígeno Leucocitário Humano do tipo DR da classe II; Ig: Imunoglobulina. Fonte: Adaptado de ABDUL-HAMID, G., 2011 e CHIARETTI, S., ZINI, G. e BASSAN, R., 2014)

De acordo com os critérios do EGIL, para caracterizar as leucemias a marcação imunofenotípica com anticorpo monoclonal deve atingir um limite mínimo de 20% para definir uma reação positiva de blastos, exceto para mieloperoxidase (MPO), CD3, CD79a e enzima nuclear deoxinucleotidil transferase terminal (TdT), as quais são considerados positivos no nível de expressão de 10% (BENE, M. *et al.*, 1995; BENE, M. C. *et al.*, 2011). Por meio disto, foi possível identificar que aproximadamente 75-80% dos casos de LLA em adultos pertencem à linhagem de células B e 20-25% à linhagem de células T (CHIARETTI, S., ZINI, G. e BASSAN, R., 2014).

No ano de 2016, a OMS apresentou uma revisão da classificação prévia das leucemias da edição de 2008 (Quadro 2), com objetivo de incorporar novos dados clínicos, prognósticos, morfológicos, imunofenotípicos e genéticos que surgiram desde a última edição (ARBER, D. A. *et al.*, 2016).

Tabela 2. Classificação das leucemias agudas/linfomas linfoblástica B e T segundo a OMS 2016.

Leucemia/linfoma linfoblástica B
Leucemia/linfoma linfoblástica B sem anormalidade genética especificada
Leucemia/linfoma linfoblástica B com anormalidades genéticas recorrentes
Leucemia/linfoma linfoblástica B com t(9;22)(q34.1;q11.2); *BCR-ABL1*
Leucemia/linfoma linfoblástica B com t(v;11q23.3); rearranjo *KMT2A*
Leucemia/linfoma linfoblástica B com t(12;21)(p13.2;q22.1); *ETV6-RUNX1*
Leucemia/linfoma linfoblástica B com hiperdiploidia
Leucemia/linfoma linfoblástica B com hipodiploidia
Leucemia/linfoma linfoblástica B com t(5;14)(q31.1;q32.3); *IL3-IGH*
Leucemia/linfoma linfoblástica B com t(1;19)(q23;p13.3); *TCF3-PBX1*
Entidade provisória: Leucemia/linfoma linfoblástica B, *BCR-ABL1-like*
Entidade provisória: Leucemia/linfoma linfoblástica B com *iAMP21*
Leucemia/linfoma linfoblástica T
Entidade provisória: Leucemia/linfoma linfoblástica T precoce
Entidade provisória: Leucemia/linfoma linfoblástica de célula *Natural Killer* (NK)

KMT2A: gene da leucemia de linhagem mista ou linfoide/mieloide; *ETV6*: gene da variante 6 do fator de transcrição transformação específica do eritroblasto (*erythroblast transformation-specific*) (ETS); *RUNX1*: fator de transcrição relacionada ao runt-1 ou gene leucemia mieloide aguda-1 (*AML1*); *IL3*: gene da interleucina-3; *IGH*: gene da imunoglobulina de cadeia pesada; *TCF3*: gene do fator de transcrição 3 (também conhecido como E2A); *PBX1*: gene homeobox 1 da leucemia de célula pré-B; *iAMP21*: gene da amplificação intracromossomal do cromossomo 21; t: translocação; v: variável. Fonte: Adaptado de ARBER, D. A. *et al.* (2016).

Na LLA do tipo B, são descritas anormalidades genéticas as quais estão associadas à características clínicas, imunofenotípicas e de prognóstico (BLOOMFIELD, C. D. *et al.*, 1986; HARBOTT, J. *et al.*, 1997; JOHANSSON, B., MERTENS, F. e MITELMAN, F., 2004; MITTELMAN, F., 1981; PUI, C.-H., ROBISON, L. L. e LOOK, A. T., 2008). Além disso, dois novos tipos de anormalidades genéticas foram incorporadas na classificação – (1) LLA tipo B com amplificação intracromossomal do cromossomo 21, associada com prognóstico adverso, o que pode resultar na necessidade de terapia mais agressiva (HARRISON, C. J. *et al.*, 2014) e (2) translocações envolvendo tirosina quinases ou receptores de citocinas ("*BCR-ABL1-like ALL*"), associada a características clínicas adversas e pior prognóstico (DEN BOER, M. L. *et al.*, 2009; MULLIGHAN, C. G. *et al.*, 2009).

Para LLA do tipo T, o perfil das anormalidades genéticas não são notoriamente associados a uma única característica biológica, e portanto, sem subdivisão conforme as alterações genéticas (VARDIMAN, J. W. *et al.*, 2009). No entanto, existe um subconjunto que é reconhecido como uma nova entidade provisória, que é a LLA do tipo célula T precoce (ETP), única subdivisão genética e imunofenotípica aceita, indicando somente a diferenciação de célula T precoce, com retenção de algumas características da célula-tronco e

da linhagem mieloide (COUSTAN-SMITH, E. *et al.*, 2009; NEUMANN, M., COSKUN, E.*, et al.*, 2013; NEUMANN, M., HEESCH, S.*, et al.*, 2013; ZHANG, J. *et al.*, 2012).

1.1.2 Fisiopatologia da LLA

Os diferentes tipos de câncer, de modo geral, compartilham uma característica biológica semelhante: a clonalidade. As alterações moleculares que são necessárias para o desenvolvimento de uma doença maligna são um fenômeno raro, considerando-se o número vasto de células-alvo suscetíveis a esta condição. Isto é, uma pequena alteração genética raramente é suficiente para o desenvolvimento do tumor maligno (GALLEGOS-ARREOLA, M. P. *et al.*, 2013).

Então, sabe-se que a LLA é derivada de lesões genéticas nas células progenitoras sanguíneas que são destinadas a sofrer diferenciação na linhagem celular T ou B. Alterações genéticas incluem mutações que transmitem a capacidade autoproliferativa, seguida de parada do desenvolvimento em um estágio específico (ARMSTRONG, S. A. e LOOK, A. T., 2005; PUI, C. H., RELLING, M. V. e DOWNING, J. R., 2004). Muitas alterações genéticas ocorrem na LLA, sendo que, na maioria dos casos infantis são alterações cromossômicas (Tabela 3) (HARRISON, C. J., 2009).

Aproximadamente 75% dos casos de LLA infantil com alterações cromossômicas são detectáveis pela análise de cariótipo, hibridização fluorescente *in situ* (FISH) e outras técnicas moleculares. A LLA de célula B inclui hiperdiploidia, hipodiploidia e pseudodiploidia, e da célula T é caracterizada pelas mutações ativadoras do gene da proteína homóloga 1 a Notch loco neurogênico (*Notch1*) e rearranjos dos fatores de transcrição, como proteína da leucemia de linhagem mista ou linfoide/mieloide (MLL). Apesar de os rearranjos genéticos demonstrarem importância nos eventos precoces da leucemogênese e serem amplamente usados no diagnóstico e estratificação de risco, ainda são insuficiente para explicar completamente o desenvolvimento da leucemia (MULLIGHAN, C. G., 2012; MULLIGHAN, C. G. e DOWNING, J. R., 2009).

Tabela 3. Anormalidades moleculares e citogenéticas na LLA.

Categoria	Citogenética	Genes envolvidos	Frequência	
			Adultos (%)	Crianças (%)
Hiperdiploidia			2-15	10-26
Hipodiploidia			5-10	5-10
Pseudodiploidia	t(9;22)(q34;q11)	*BCR-ABL1*	15-25	2-6
	del(9)(q21-22)	*p15,p16*	6-30	20
	t(4;11); t(9;11); t(11;19); t(3;11)	*MLL*	5-10	<5
	del(11)(q22-23)	*ATM*	25-30	15
	t(12;21)(p12;q22)	*TEL-AML1*	<1	20-25
	t(1;19)	*E2A-PBX1*	<5	<5
	t(17;19)	*E2A-HLF*	<5	<5
	t(1;14)(p32;q11)	*TAL1*	10-15	5-10
	t(7;9)(q34;q32)	*TAL2*	<1	<1
	t(10;14)(q24;q11)	*HOX11*	5-10	<5
	t(5;14)(q35;q32)	*HOX11L2*	1	2-3
	t(1;14)(p32;q11)	*TCR*	20-25	20-25
	del(13)(q14)	*miR15/miR16*	<5	<5
	t(8;14); t(8;22); t(2;8)	*C-MYC*	5	2-5
	+8	*?*	10-12	2
	del(7p)	*?*	5-10	<5
	del(5q)	*?*	<2	<2
	del(6q); t(6;12)	*?*	5	<5

MLL: também conhecido como *KMT2A*; *ATM*: gene da ataxia telangiectasia mutada; *TEL*: também conhecido como *ETV6*; *AML1*: também conhecido como *RUNX1*; *HLF*: fator hepático da leucemia; *TAL*: gene da leucemia linfoblastica aguda de célula T 2; *HOX*: gene *homeobox*; *TCR*: gene do receptor de célula T; *miR*: gene do microRNA; *C-MYC*: gene da mielocitomatose celular; del: deleção; +8: trissomia no cromossomo 8; p: braço curto do cromossomo; q: braço longo do cromossomo. Fonte: Adaptado de JABBOUR, E. *et al.* (2015).

As modificações epigenéticas têm demonstrado um papel fundamental na influência da expressão gênica (DEATON, A. M. e BIRD, A., 2011; EASWARAN, H., TSAI, H. C. e BAYLIN, S. B., 2014). A análise de perfis de metilação de DNA em pacientes pediátricos LLA de célula B mostraram hipermetilação da região promotora como uma característica proeminente, afetando genes previamente implicados na leucemia e em outras doenças hematológicas (WONG, N. C. *et al.*, 2012).

Outro mecanismo epigenético importante é a atuação dos miRNAs, que são reguladores epigenéticos, tendo o RNA mensageiro (mRNA) como alvo específico para modular os padrões da expressão gênica e vias de sinalização celular. O perfil de expressão dos miRNAs no câncer pode predizer o prognóstico do paciente e a resposta ao tratamento (BOUCHIE, A., 2013; HAYES, J., PERUZZI, P. P. e LAWLER, S., 2014; STAHLHUT, C. e SLACK, F. J., 2013). Além disso, os miRNAs estão sendo usados para diferenciar as linhagens de células B e T (YEH, C. H., MOLES, R. e NICOT, C., 2016). Por conseguinte, pode-se

deduzir que a fisiopatologia da LLA envolve mecanismos genéticos e epigenéticos, como a regulação por miRNAs.

O estudo destes fatores pode identificar marcadores na progressão da doença e um direcionamento ao tratamento.

1.1.3 Aspectos Clínicos da Doença

Os blastos leucêmicos primeiramente infiltram a MO, ocupando mais de 20% (pela OMS) ou mais de 30% (pelos critérios da FAB) do total de células nucleadas, chegando a 80-100% de ocupação. A primeira consequência é a supressão da hematopoiese normal, sendo que a expansão destes blastos ocupa o espaço necessário à produção fisiológica das células hematológicas na medula, acarretando deficiência das outras linhagens celulares como hemácias, plaquetas e neutrófilos, causando anemia, trombocitopenia e/ou leucocitose com presença de blastos. Os blastos anormais também secretam fatores inibitórios e indutores de fibrose, tornando a disfunção medular mais grave do que o esperado apenas pela simples ocupação de espaço (LORENZI, T. F., 2006). Os blastos neoplásicos são lançados na corrente sanguínea, justificando o termo leucemia (leucócitos), e eventualmente atingem um número suficiente para determinar uma leucocitose. Uma vez na corrente sanguínea, os blastos podem então infiltrar os órgãos, com uma preferência para os linfonodos, baço, fígado, gengiva, órbita, sistema nervoso central e testículos (LORENZI, T. F., 2006).

A apresentação clínica na LLA acontece de forma súbita. Geralmente, os pacientes apresentam dispneia, fadiga ou sangramento espontâneo, indisposição, letargia, perda de peso, febre e sudorese noturna de uma forma mais frequente. Além disso, podem aparecer linfadenopatia, esplenomegalia e/ou hepatomegalia. As neuropatias cranianas e infiltração testicular (aproximadamente 2% do gênero masculino) podem ocorrer devido às recidivas ou recaídas extramedulares, enquanto náusea, vômito, cefaleia ou papiledema são resultantes de infiltração meníngea e/ou obstrução do fluído do líquor (BORIM, L. N. B. *et al.*, 2000; CORNACCHIONI, A. L. B. *et al.*, 2004; FARHI, D. C. e ROSENTHAL, N. S., 2000; HAMERSCHLAK, N., 2008; LARSON, R. A. e ANASTASI, J., 2008; LOCATELLI, F. *et al.*, 2012; MEDEIROS, G. E. B. *et al.*, 2004).

Os pacientes podem apresentar dores ósseas em 25% dos casos, com lesões ósseas, osteopenia difusa, dores lombares ou artrite assimétrica e hemorragia óssea, como consequência da infiltração leucêmica pelos blastos (GUR, H. *et al.*, 1999; IKEUTI, P. S., BORIM, L. N. B. e LUPORINI, R. L., 2006).

1.1.4 Diagnóstico Laboratorial

Quanto ao diagnóstico inicial das leucemias, é realizado um exame físico do paciente, seguido de análise quantitativa e qualitativa do sangue total por meio do hemograma e confecção da extensão (esfregaço) sanguínea. Para confirmação do diagnóstico são realizados punção e biópsia de MO, por imunofenotipagem e análise citogenética (MANISHA, P., 2012; PUI, C. H. *et al.*, 2004).

No hemograma de pacientes suspeitos, em especial de LLA, é observado anemia normocítica e normocrômica, contagem diminuída de reticulócitos, trombocitopenia e comumente leucocitose associada à presença significativa de blastos (ALMEIDA, T. J. B., 2009; FARIAS, M. G. e CASTRO, S. M. D., 2004; FERRI, F. F., 2012; VERAS, G., ARAGÃO, V. M. D. F. e SANTOS, A. M. D., 2012).

Após análise do hemograma, é solicitado o mielograma para estimar a porcentagem de blastos na microscopia pela punção aspirativa da MO na região lombar. A caracterização de mais de 20% de blastos, megacariócitos diminuídos ou ausentes e células precursoras mieloides e eritróides com aspectos normais são confirmatórios para a LLA (ALMEIDA, T. J. B., 2009; FARIAS, M. G. e CASTRO, S. M. D., 2004; REGO, E. M. e SANTOS, G. A. S., 2009).

A imunofenotipagem por citometria de fluxo é um método utilizado como ferramenta padrão e indispensável na clínica para diagnóstico diferencial, classificação, estadiamento e monitoramento da doença residual mínima (DRM) das leucemias agudas e síndromes mielodisplásicas. Com o uso de anticorpos monoclonais é possível identificar diferentes linhagens celulares e determinar o grau de maturação celular (CRAIG, F. E. e FOON, K. A., 2008; ORFAO, A. *et al.*, 2004). Por meio desses recursos, o direcionamento para o tratamento acaba sendo mais eficaz evitando a falha terapêutica.

1.1.5 Fases Clínicas e Classificação de Risco da LLA

O tratamento da LLA normalmente é dividido em três fases – a fase de indução, intensificação (ou consolidação) e manutenção. A fase de indução da remissão completa é definida como restabelecimento da hematopoiese normal em que, espera-se conduzir a um estado com menos de 5% de blastos na MO e ausência de qualquer sinal ou sintoma da doença. Quando esta fase é concluída, os pacientes ainda podem apresentar graus variados de leucemia residual e alguns podem portar até 10 bilhões de células leucêmicas

(CAMPANA, D. e PUI, C. H., 1995; CAZÉ, M. O., BUENO, D. e SANTOS, M. E. F. D., 2010; PUI, C. H. e CAMPANA, D., 2000).

Após o restabelecimento da hematopoiese normal, os pacientes se tornam candidatos para terapia de intensificação, a qual se refere à readministração do regime terapêutico da indução ou administração de altas doses de agentes quimioterápicos que não foram usadas na fase de indução, com objetivo de diminuir o número de células tumorais a níveis muito baixos ou eliminá-las (KAUSHANSKY, K. *et al.*, 2016).

A fase final do tratamento pode durar 2 anos. Os acúmulos de altas concentrações intracelulares de metabólitos ativos dos medicamentos e a administração combinada aos limites de tolerância têm sido associados com melhora no desfecho clínico (CHESSELLS, J. M. *et al.*, 1997; SCHMIEGELOW, K. *et al.*, 1995). Na fase de manutenção, o objetivo é eliminar resíduos de células leucêmicas, pois estas têm o potencial de recidivar a doença, porém não interferem no desenvolvimento das células sanguíneas normais (INABA, H., GREAVES, M. e MULLIGHAN, C. G., 2013).

O Grupo Brasileiro de Tratamento de Leucemia na Infância (GBTLI), atualizado em 2009, classifica os pacientes em dois grandes grupos de risco, de acordo com parâmetros hematológicos e a resposta terapêutica (Tabela 4). Assim, a determinação de um protocolo de tratamento diferenciado para cada grupo de risco acaba se tornando viável. De acordo com o protocolo do GBTLI, os pacientes classificados como baixo risco durante o tratamento e que apresentem requisitos para inclusão do grupo de alto risco, devem ser reclassificados (CAZÉ, M. O., BUENO, D. e SANTOS, M. E. F. D., 2010; INCA, 2001).

Tabela 4. Classificação de risco na LLA.

	Baixo Risco	**Alto Risco**
Idade	≥1 ano a <9 anos	<1 ano e >9 anos
Leucometria ao diagnóstico	<50.000/mm^3	≥50.000/mm^3
Leucometria no 7º dia de tratamento	<5.000/mm^3	≥5.000/mm^3 *
Blasto no sangue periférico e comprometimento medular no 14º dia de tratamento	Ausência de blastos e baixo comprometimento medular	Presença de blastos e/ou comprometimento medular extenso
Medula no 28º dia de indução	Baixa contagem de células leucêmicas	Evidência de acometimento extramedular ao final da indução
Comprometimento do SNC	Ausência de blastos no líquor no 14º dia de tratamento	Persistência de blastos leucêmicos

*Respondedores lentos ao tratamento; SNC: Sistema Nervoso Central. Fonte: CAZÉ, M. O., BUENO, D. e SANTOS, M. E. F. D., 2010 e INCA, 2001.

Variações interindividuais na resposta ao tratamento de pacientes com câncer constituem um sério problema e, até o momento, não existem biomarcadores que possam predizer qual grupo responderá positivamente e quais indivíduos não irão responder ou sofrerão os efeitos adversos da presença das drogas no organismo, o que torna o estudo de genes metabolizadores de grande valia para a oncologia. Na LLA, muitos estudos têm sido realizados na busca por marcadores que possam estar relacionados à suscetibilidade ou influenciar na resposta à quimioterapia (MROZEK, K., HARPER, D. P. e APLAN, P. D., 2009; ZHANG, C. *et al.*, 2015).

Dentro de uma espécie, os cromossomos homólogos são bastante similares entre si, mas em determinadas localizações do cromossomo (*loci*) pode haver variabilidade na sequência do DNA. Se a variação é encontrada em uma frequência superior a 1% da população, denomina-se polimorfismo (BALASUBRAMANIAN, S. P. *et al.*, 2004). Essas alterações podem ocorrer tanto nas sequências codificadoras do gene, quanto nas sequências não codificadoras, sendo que ambas podem levar à produção deficitária e/ou de proteínas defeituosas (LODISH, H. *et al.*, 2000). Por exemplo, a investigação de polimorfismos de base única (SNPs) em genes candidatos aponta que o risco de leucemias, inclusive LLA, pode ser atribuído a polimorfismos em genes relacionados ao metabolismo de xenobióticos e reparo de DNA (CHOKKALINGAM, A. P. e BUFFLER, P. A., 2008; URAYAMA, K. Y. *et al.*, 2013).

Estudos epidemiológicos evidenciam que mais de 90% das neoplasias estão relacionadas a fatores ambientais, como alimentação e tabaco, e que diversos componentes químicos podem interagir com as células, modificando macromoléculas e iniciando o processo de tumorigênese. Durante o processo evolutivo foram desenvolvidos vários mecanismos biológicos de proteção a estes componentes tóxicos. A biotransformação de drogas é um deles, onde o fígado realiza um processo enzimático que intercepta xenobióticos, transformando-os em substâncias mais hidrossolúveis e facilmente excretadas pelo organismo, impedindo possíveis danos ao material genético (LOSI-GUEMBAROVSKI e CÓLUS, 2001).

Os xenobióticos são substâncias químicas alheias ao sistema biológico com as quais o homem tem contato, tais como medicamentos, produtos industriais, alimentos, pesticidas, cosméticos, poluentes, entre outros. Dentre as variantes genéticas do metabolismo de xenobióticos que têm sido associadas de forma mais consistente com o aumento do risco de câncer incluem-se a família das Glutationa S-transferases (GSTs) e da NAD(P)H oxiredutases (NQO1) (SHASTRY, B. S., 2006).

Em suma, a LLA é um tipo de câncer que demonstra uma complexidade desde a patogênese até o estabelecimento do diagnóstico e tratamento. Além disso, vários fatores

intrínsecos podem influenciar no curso da doença. Desta maneira, há necessidade de investigar genes envolvidos na metabolização de drogas utilizadas nestes pacientes e que podem estar envolvidos na resposta terapêutica.

1.2 GLUTATIONAS GSTT1 E GSTM1

As GSTs desempenham um papel importante no sistema de proteção celular. A super família das GSTs são codificadas por cinco classes de genes μ, θ, π, α, σ e são enzimas envolvidas na fase II da biotransformação, qualificada para converter compostos endógenos lipossolúveis em substratos hidrossolúveis. Essas enzimas são implicadas na detoxificação de uma ampla gama de compostos, incluindo xenobióticos, pesticidas, carcinógenos ambientais, hidrocarbonetos policíclicos aromáticos (PAHs) e alguns quimioterápicos (incluindo agentes alquilantes, doxorrubicina e vincristina). Polimorfismos funcionais foram relatados em pelo menos três dos genes que codificam GSTs, incluindo *GSTM1, GSTT1* e *GSTP*. Os genes *GSTT1* e *GSTM1* exibiram um maior grau de polimorfismo, sendo um deles a deleção completa do gene que leva a perda da atividade enzimática (ALVES, S. *et al.*, 2002).

As enzimas GSTs, principalmente a codificada pelo gene *GSTM1*, metaboliza o benzopireno e outros produtos de hidrocarbonetos policíclicos aromáticos. A enzima codificada pelo gene *GSTT1* parece ser a principal envolvida na biotransformação de toxinas de baixo peso molecular, como óxido de etileno e outros substratos considerados carcinógenos (LINHARES et al., 2006; LOSI-GUEMBAROVSKI et al., 2008).

A família *GSTs* pode modular o risco de leucemia por meio de dois mecanismos potenciais: mediando o metabolismo específico de leucemogênese ou afetando diretamente o potencial redox na célula, protegendo o DNA contra danos induzidos por radicais livres (DUNNA, N. R. *et al.*, 2013).

Além disso, as enzimas dessa família são capazes de regular a citotoxicidade de uma variedade de drogas quimioterápicas (HOBAN, P. R. *et al.*, 1992), e essas enzimas catalisam a conjugação de moléculas eletrofílicas tóxicas e cancerígenas com a glutationa protegendo as macromoléculas dos danos celulares (BOYER, T. D., 1989).

As enzimas codificadas por esses genes desempenham um passo importante na desintoxicação de xenobióticos e a ausência de sua atividade pode levar ao aumento da toxicidade e suscetibilidade ao desenvolvimento de tumores malignos. Estudos detectaram uma alta frequência de genótipo nulo para esses dois genes em indivíduos com cânceres diferentes, principalmente câncer de pulmão, bexiga e cavidade oral. Também demonstraram um risco

aumentado de desenvolver câncer de cabeça e pescoço em indivíduos com deleções no gene *GSTT1*. A deleção do gene *GSTM1* também foi correlacionada com outras doenças relacionadas ao tabagismo, como arteriosclerose e enfisema pulmonar (CASCORBI, I., 2006; OLSHAN, A. F. *et al.*, 2000)

Suspeita-se que pacientes com um genótipo nulo de *GST* exibem desintoxicação prejudicada de agentes genotóxicos ambientais e drogas quimioterápicas, levando a um risco aumentado de desenvolver cânceres primário e secundário e complicações relacionadas ao tratamento (KRAJINOVIC, M. *et al.*, 1999; SAADAT, I. e SAADAT, M., 2000).

1.3 Família das NAD(P)H oxiredutases – gene *NQO1*

O estresse oxidativo é uma condição biológica na qual acontece um desequilíbrio entre a produção de espécies reativas de oxigênio (EROs) e a sua remoção ou eliminação. O sistema de remoção ou eliminação de EROs podem ou não envolver mecanismos enzimáticos que conseguem remover ou reparar os danos causados por essas espécies reativas. Essa condição biológica muitas vezes pode desempenhar um papel importante na etiologia do câncer (HONG, C. C. *et al.*, 2007). Portanto, um balanço entre os oxidantes e antioxidantes endógenos é comumente afetado pela variação nos genes envolvidos com a geração e a remoção das espécies reativas (CAI, Q. *et al.*, 2004; TAMIMI, R. M. *et al.*, 2004).

As quinonas são uma classe de compostos orgânicos derivados de hidrocarbonetos aromáticos, encontradas em vários sistemas biológicos, sendo componentes da cadeia transportadora de elétrons (MONKS, T. J. *et al.*, 1992; MONKS, T. J. e JONES, D. C., 2002). Envolvida no metabolismo das quinonas, a enzima NAD(P)H: quinona oxidorredutase 1 (NQO1) é uma redutase obrigatória de dois elétrons que participa na proteção contra efeitos tóxicos de drogas anticancerígenas. Além disso, essa enzima realiza a ativação de certas quinonas antitumorais (ROSS, D. *et al.*, 2000) e está envolvida na defesa contra formas reativas de oxigênio (NIOI, P. e HAYES, J. D., 2004).

A NQO1 está envolvida na proteção contra o estresse oxidativo e a carcinogênese por catalisar reações bioredutoras e bioativadoras (SIEGEL, D. *et al.*, 2004; TSENG, L. M. *et al.*, 2009), incluindo a estabilização do supressor tumoral *TP53* (ANWAR, A. *et al.*, 2003; ASHER, G., LOTEM, J., KAMA, R.*, et al.*, 2002; ASHER, G., LOTEM, J., SACHS, L.*, et al.*, 2002).

Foi descoberto que o gene que codifica para esta enzima, o gene *NQO1,* possui um polimorfismo genético de base única (C609T) (rs1800566) localizado no éxon 6, o qual origina uma mudança na sequência de aminoácidos (Pro187Ser) (ROSS, D. *et al.*, 1996; TRAVER, R. D. *et al.*, 1992; TRAVER, R. D. *et al.*, 1997). Esta mutação ocasiona a perda de sua função, onde indivíduos homozigotos recessivos (TT) não demonstram seus efeitos citoprotetores, enquanto heterozigotos (CT) apresentam baixa atividade da enzima (LANCIOTTI, M. *et al.*, 2005).

Este polimorfismo tem sido associado ao risco de desenvolvimento de diversos tipos de câncer (MALIK, M. A., ZARGAR, S. A. e MITTAL, B., 2011; YUAN, W. *et al.*, 2011; ZHANG, J. *et al.*, 2003), incluindo também LLA (KRACHT, T. *et al.*, 2004; ZHOU, Y. *et al.*, 2014). Isso sugere que a enzima NQO1 está relacionada ao metabolismo de drogas antitumorais e a variante alélica modifica sua atividade. Também foi verificado em um estudo relacionando as alterações na resposta a terapia adjuvante com doxorrubicina e ciclofosfamida, com ou sem tamoxifeno, que o genótipo polimórfico TT estava associado com pior evolução clínica e baixa probabilidade de sucesso no tratamento, demonstrando um possível efeito modulador desse polimorfismo na eficácia da terapia adjuvante do câncer de mama (JAMIESON, D. *et al.*, 2011).

De um modo geral, existe a necessidade de se ampliar os estudos envolvendo marcadores moleculares responsáveis por diferentes etapas do metabolismo de xenobióticos, drogas e compostos endógenos, e, sobretudo, avaliar a presença destas mutações em relação a parâmetros de prognóstico, progressão e resposta terapêutica na LLA.

2 OBJETIVOS

2.1 OBJETIVO GERAL

Avaliar os polimorfismos *GSTs e NQO1* e os níveis plasmáticos da glutationa em pacientes infantojuvenis com leucemia linfoide aguda.

2.2 OBJETIVOS ESPECÍFICOS

- Analisar as frequências genotípicas e alélicas dos polimorfismos *GSTs e NQO1* em pacientes com LLA e em indivíduos controle livre de neoplasias;
- Avaliar possíveis associações entre a presença das variantes genéticas propostas entre os dois grupos e o risco de suscetibilidade à doença num estudo do tipo caso-controle;
- Correlacionar a presença dos polimorfismos propostos em pacientes com LLA infantojuvenis com o risco de recidiva e óbito;
- Determinar os níveis plasmáticos de GST em pacientes com LLA e grupo controle;
- Comparar a presença dos polimorfismos estudados com os níveis plasmáticos de glutationa nos grupos caso e controle.

3 MATERIAIS E MÉTODOS

3.1 COLETA DE AMOSTRAS

O presente trabalho foi cadastrado na Plataforma Brasil e todos os procedimentos foram aprovados pelo Comitê de Ética em Pesquisa com Seres Humanos da Universidade Estadual de Londrina (CEP/UEL 189/2013 – CAAE Nº 17123113400005231). Todos os participantes e responsáveis legais pelas crianças foram orientados e somente foram utilizadas amostras de pacientes autorizados por seus responsáveis, mediante a assinatura do Termo de Consentimento Livre e Esclarecido.

Neste trabalho foram coletados 5mL do sangue periférico de 74 pacientes de 0 a 19 anos diagnosticados com LLA atendidos no Serviço de Oncopediatria do Hospital do Câncer de Londrina (HCL), sendo classificados em alto e baixo risco de acordo com o GBLT-LLA 2009, e 115 controles com faixa etária similar, os quais não possuíam histórico de neoplasia, processos inflamatórios e infecciosos, coletados no Hospital Universitário da Universidade Estadual de Londrina.

3.2 EXTRAÇÃO DE DNA

A partir do sangue periférico coletado em tubo EDTA (ácido etilenodiamino tetra-acético), foi extraído o DNA utilizando o kit comercial *Biopur Mini Spin Plus Kit* (*Biometrix Diagnostica*, Curitiba, Brasil) segundo as especificações do fabricante. O DNA extraído foi eluído em 50 µL de tampão de eluição e quantificado por espectrofotometria no aparelho *NanoDrop 2000c*® (*Thermo Fisher Scientific*, Wilmington, DE, EUA). A absorbância no comprimento de onda de 260 nm foi utilizada para determinar a concentração de ácidos nucleicos, e a razão para determinar o grau de pureza em relação a proteínas foi obtida entre as absorbâncias 260 e 280nm.

3.2.1 Análise de presença/ausência em homozigose dos genes *GSTM1* e *GSTT1*

A reação de co-amplificação em cadeia pela polimerase foi realizada baseada no protocolo de PCR *Multiplex* de ABDEL-RAHMAN et al. (1996), modificado, nas seguintes condições: tampão da enzima (20 mM de tris-HCl pH 8,4; 50 mM de KCl); 2 mM de $MgCl_2$; 10 pmol de cada iniciador; 1,25 U de Taq DNA polimerase; 2 mM de dNTPs; 100ng de DNA genômico total e água ultrapura estéril para completar o volume final.

Os fragmentos foram amplificados em termociclador PTC-100 (MJ *Research, Inc*) e submetidos à eletroforese (3V/mL). Os produtos de PCR foram submetidos a eletroforese em gel de poliacrilamida 10% e visualizados através da técnica de coloração por nitrato de prata.

Os iniciadores para o gene *CYP1A1* amplificam um fragmento não polimórfico de 312 pb, usado como controle interno da reação. Os fragmentos de 215 e 480 pb foram observados, respectivamente, nos indivíduos *GSTM1* e *GSTT1* positivos. A ausência de amplificação *GSTM1* (215 pb) ou *GSTT1* (480 pb), na presença de controle interno, indicou os respectivos genótipos nulos para cada gene, ou para ambos.

3.2.2 Análise do polimorfismo rs1800566 do gene *NQO1*

Para a análise do polimorfismo rs1800566 do gene *NQO1* foi realizada a amplificação por PCR e subsequente análise do fragmento de restrição (RFLP).

O PCR foi realizado em volume final de 12,5uL contendo 100ng de DNA como template, 1,25µL de *buffer* 10X, 1 unidade de *Taq*-DNApolimerase (Invitrogen™, Carlsbad, USA), 100 µmol de dNTP e 10pmol de cada iniciador.

A amplificação foi avaliada por eletroforese em gel de poliacrilamida 10% (174pb). Em seguida, os produtos foram submetidos à digestão enzimática com 1 unidade da enzima *Hinf*I (Boehringer, Mannheim, Germany) e visualizados após a eletroforese em gel de poliacrilamida 10% corado com nitrato de prata.

O alelo prevalente demonstrou um produto de 174pb resistente à digestão enzimática, enquanto o alelo raro T (polimórfico) revelou um fragmento de 119pb e outro de 55pb.

3.2.3 Quantificação da glutationa plasmática

Alíquotas de 100 µL de plasma foram desproteinizadas com 100 µL de ácido tricloroacético a 50% e centrifugadas por 5 minutos a 4000 rotações por minuto. Um volume de 50 µL deste sobrenadante foi adicionado à microplaca e misturado com 200 µL de solução tampão Tris-HCl (0,4 molar; pH 8,9). Para controlar a reação, uma curva padrão com diluição em série da solução 0,03 M de GSH (Sigma) foi realizada. Finalmente, 50 µL de ácido 5,5`-ditiobis (2-nitrobenzóico) (DTNB) 0,01 M (preparado em metanol) foram adicionados a todas na curva padrão e nas amostras, e a detecção foi realizada por espectrofotometria com comprimento de onda de 405nm.

3.3 ANÁLISE ESTATÍSTICA

As frequências dos genótipos dos polimorfismos foram submetidas ao teste qui-quadrado para verificar o equilíbrio de *Hardy-Weinberg*. Os estudos de associação do tipo caso-controle foram analisados pelo cálculo da *Odds Ratio* (OR) com intervalo de confiança (IC) a 95% e por teste de *Fisher*. Toda a análise estatística foi realizada usando o software *Prism 6* (*GraphPad Software*, San Diego, EUA). Os testes Tau_b de Kendall foram utilizados na comparação entre os parâmetros analisados em relação aos diferentes genótipos, através do programa estatístico SPSS (versão 20). Valores de $p< 0.05$ foram considerados significantes.

4 PRODUÇÃO CIENTÍFICA

4.1 Artigo 1 - Review

ACUTE LYMPHOID LEUKEMIA ETIOPATHOGENESIS

Abstract

Acute lymphoid leukemia (ALL) is a type of hematological neoplasm that affects the precursor cells of strains B and T, with a higher incidence in the pediatric range. Its pathophysiology involves the blockade of differentiation and the high proliferation of precursor cells, called leukemic blasts. Despite the lack of information in the literature, it is believed that leukemogenesis originates from a complex interaction between environmental and genetic factors, which combined lead to cellular modifications. The pathophysiology of ALL involves genetic and environmental mechanisms at different levels, and also there must be a complex relationship between these factors. Environmental factors have been evaluated as possible predisposing factors in the development of ALL but there are still conflicting results in the world literature. In this context, the aim of the present review is to discuss the major exogenous factors regarding ALL.

Keywords. Leukemia, Childhood, Infection, Environment

Introduction

Childhood and juvenile cancer, which affects individuals between 0 and 19 years old, consists of a set of diseases that have their own characteristics in relation to the cells that make up the tumors (histological type) and clinical behavior of the disease (Little 1999). In most populations, childhood and juvenile cancer accounts for 1% to 4% of all malignant tumors, and in developing countries, where the child population reaches 50%, this proportion of childhood cancer accounts for 3% to 10% of all cancers. In developed countries, this proportion decreases to around 1% (ACS 2016). Among childhood cancer types worldwide, leukemia is common in most populations, corresponding to 25% to 35% of cases (Howlader, Noone et al. 2014).

Leukemias are different groups of hematologic diseases with a different biological concept, clinical presentation, prognosis and treatment response, characterized by the presence

of an abnormal cell population suppressing the normal production of cellular components of the hematopoietic system (Polychronakis, Dounias et al. 2013).

The origin of leukemias comes from hematopoietic stem cells (HSCs) and precursors in the bone marrow (BM), promoting proliferation and infiltration of leukemic cells (Konopleva and Jordan 2011, Azizidoost, Babashah et al. 2014). In general, hematological malignancies are classified according to lineage, degree of maturation and form of cellular involvement in the BM. Myeloid lineage neoplasms may include granulocytes (neutrophils, eosinophils, basophils), monocytes, erythrocytes, platelets (megakaryocyte derivatives) and mast cells. In contrast, the lymphoid lineage corresponds to B or T lymphocyte and natural killer (NK) cells (Vardiman, Thiele et al. 2009).

The pathophysiology of acute lymphoid leukemia (ALL) involves complex genetic and environmental mechanisms at different levels, and also there must be a complex relationship between these factors. It is believed that ALL may originate from interactions between exogenous factors. Therefore, this review proposes to clarify the understanding of ALL regarding environmental factors.

Acute lymphoid leukemia

ALL is a malignant disorder that originates from a single haematopoietic precursor affecting the B or T cell line. These cells can acquiring a series of genetic alterations that could disrupts normal maturation processes, leading to blockade of differentiation and to transformed cell proliferation (Graux 2011).

The incidence of this disease varies worldwide. However, there is a higher frequency in countries with high socioeconomic development, except for some cases of ALL reported in some Hispanic cities, Costa Rica and Mexico City, that also shows high frequency of these malignancy (Mejia-Arangure, Bonilla et al. 2005, Mejia-Arangure, Perez-Saldivar et al. 2011).

In the United States, have been estimated approximately 6000 new ALL cases, at a ratio of 1.3 men to 1 woman (Siegel, Naishadham et al. 2012). Most patients are children, with 60% of cases occurring in individuals under 20 years old (Pui, Robison et al. 2008, Stanulla and Schrappe 2009, Hunger, Lu et al. 2012).

ALL, like cancer in general, probably originates from interactions between exogenous and endogenous exposure and genetic susceptibility (Inaba, Greaves et al. 2013). ALL pathogenesis occurs from important genetic lesions in genes involved with T or B lymphocyte differentiation (Pui, Robison et al. 2008). The challenge is to identify exposure elements and

relevant inherited genetic variants, and decipher how and when these factors contribute to the multi-stage natural history of ALL from initiation (usually in the womb) to disease manifestation (Greaves and Wiemels 2003).

The classification for ALL B-cells precursor is based of cell surface expression molecules related to normal cell maturation. The markers that allow this definition are identified through immunophenotypic assay by flow cytometry and the recommend criteria of identification consist at least two specific antigens from each lineage.

The European Group for the Immunological Characterization of Leukemia (EGIL) simplified the characterization of ALL, standardizing the different nomenclatures used by different researchers in the field of cellular immunophenotyping (Bene, Castoldi et al. 1995).

In all ALL B-cell precursors, the cells are generally positive for CD79, TdT, HLA-DR, CD19 and CD22. This group is subdivided into pro-B ALL (CD10-/ cIgμ-), common ALL (CD10 +/ cIgμ-) and pre-B ALL (CD10 +/ cIgμ +/ sIgμ +/-). In the pre-B subtype, the cells are positive for CD19, CD24, HLA-DR, cCD22 and CD10. TdT and CD20 antigens present variable expression. Its characteristic is the presence of cytoplasmic μ heavy chain (Szczepanski, van der Velden et al. 2006). Mature B-cell ALL represents 2 to 5% of ALL. The cells have the CD19, CD20, CD22, CD24 and sIgμ antigens. Many cases are CD10 + (Szczepanski, van der Velden et al. 2006). Flow cytometry can also be used to classify ALL according to with its DNA content where ALL are classified as hyperdiploid, implying a good prognosis, and hypodiploids, with an unfavorable prognosis (Jennings and Foon 1997, Szczepanski, van der Velden et al. 2006).

Pathophysiology of ALL

Leukemia and other cancers share the same biological characteristic which is clonality. Molecular changes required for cancer development are a rare phenomenon, given the large number of target cells susceptible to this condition. Normally, a single genetic change is rarely enough for malignant tumor development (Gallegos-Arreola, Borjas-Gutiérrez et al. 2013).

The development of a malignant hematological disease probably involves a mutation in a critical gene of cell proliferation, differentiation and/or survival in a hematopoietic progenitor (Greaves 2002). Most leukemia mutations are acquired and occur in a lymphoid cell progenitor; mutated genes are less often inherited (1 to 5% of leukemias), and this involves a numerical chromosomal abnormality, such as 21 trisomy (Seewald, Taub et al. 2012).

When an oncogene is activated by mutation, the encoded protein is structurally modified and generally has increased transformative activity, remaining in its active state, and continuously transmitting its signals through the interaction of tyrosines and/or threonine kinases. These signals induce incessantly continued cell proliferation (Gallegos-Arreola, Borjas-Gutiérrez et al. 2013).

There are mutations that suppress gene function and occur in tumor suppressor genes, such as *TP53*. However, less than 3% of childhood patients and an average of 9% of adult ALL patients have mutations in *TP53* (Chiaretti, Zini et al. 2014).

Numeric chromosomal abnormalities as well as structural rearrangements (translocations) commonly occur in ALL. Significant cytogenetic abnormalities in B-cell precursors are associated with poor prognosis ALL, including t(9; 22) or Philadelphia chromosome, with frequency directly proportional to age (Rix, Colinge et al. 2013); t (4; 11), related to the mixed lineage leukemia (MLL) gene, common in childhood and also associated with myeloid leukemia (Armstrong, Staunton et al. 2002, Armstrong, Kung et al. 2003); hypodiploidy (Nachman, Heerema et al. 2007); and chromosome 8 trisomy in adults diagnosed with ALL (Bakshi, Brahmbhatt et al. 2012).

In addition, the genetic alterations associated with ALL are mainly localized to sites where oncogenes exist, for example: chromosome 9 GLA oncogene, which is affected by certain translocations in ALL (Cazzaniga, van Delft et al. 2011); the MLL oncogene MLL related to ALL in children (Ayton and Cleary 2001); t (8; 14) translocation associated with gene dysregulation of the C-MYC oncogene (Palomero, Lim et al. 2006); *TP53* tumor suppressor gene mutations (Vilas-Zornoza, Agirre et al. 2011); and deletions and inversions, such as the transcription factor *PAX5* deletions, present in at least 30% of B-cell precursor ALL (Heltemes-Harris, Willette et al. 2011).

Aberrant methylation of CpG islands in gene promoter regions has been identified in ALL cell lines and is considered important since methylation of CpG dinucleotides near transcription initiation sites may silence gene expression (Feinberg and Tycko 2004). Thus, hypermethylation of tumor suppressor genes and hypomethylation of oncogenes can trigger leukemias.

Another important mechanisms of ALL development are the modification of angiogenesis (Schneider, Dubus et al. 2011), signal transduction in interaction with tyrosine kinase receptors and, finally, apoptosis regulatory molecules (Sanda, Tyner et al. 2013), as is the case of the *BCL2* gene, which encodes a cytoplasmic protein located in mitochondria and increases cell survival by inhibiting apoptosis.

There are cases of secondary hematologic malignancies, developed from complications of cancer treatment. They usually manifest as acute leukemias or myelodysplastic syndromes and are frequently high, possibly due to increased use of genotoxic agents in antitumor therapies and increased survival in other cancers (Smith, McCaffrey et al. 1996).

Increasing of evidence support a multi-step process in leukemogenesis, with sequential steps and a series of changes in oncogenes, tumor suppressor genes and microRNA genes in tumor cells (Greaves 2002, Croce 2008). Unlike genes involved in cancer development, genes for microRNAs do not encode proteins; its products are small RNA molecules (single stranded 21-23 nucleotides) that recognize and bind to messenger RNA (mRNA) nucleotide sequences, blocking the translation of the protein, thereby regulating gene expression (Calin, Dumitru et al. 2002, Croce 2008).

Several microRNAs have been implicated in the pathogenesis of ALL (Akbari Moqadam, Lange-Turenhout et al. 2013, Benetatos and Vartholomatos 2013, Dou, Li et al. 2013). In this context, Li, Li et al. (2013) have identified an increase in miR-708, miR210 and miR-181b microRNA expression in B-line precursor ALL cells (common ALL). Moreover, they demonstrated that miR708 expression is related to the high-risk ALL group, when compared to low-risk groups, by regulating the expression of the ciliary neurotropic factor receptor (*CNFTR*), neuronatin (*NNAT*) and guanine nucleotide-binding protein subunit gamma *(GNG12)* genes.

Molecular changes required for leukemia development are rare phenomena when considering the large number of target cells susceptible to genetic modification (Greaves 2002). It is important to mention that when referring to the origin of cancer, in the case of ALL, two terms must be referenced: the original cell and the leukemic stem cell.

Environmental factors

It is believed that ALL may originate from interactions between exogenous and endogenous exposures, genetic (inherited) or random susceptibility (Greaves and Wiemels 2003, Inaba, Greaves et al. 2013).

Although little is known about the etiology of ALL, the multifactorial behavior of the disease suggests that risk factors contribute to its development, whether by ionizing radiation, chemotherapy and/or chromosomal abnormalities (Han, Lan et al. 2010). On the other hand, there are three highlighted hypotheses: population mix (Strachan 1989), late infection (Kinlen

1995) and hygienic-sanitary (Greaves 2006), suggesting the involvement of the immune system in the etiology of ALL.

Some factors associated with an increased risk for ALL were identified. One of the most related environmental factors is the involvement of ionizing radiation, which is more prevalent in pediatric leukemias, especially in ALL and acute myeloid leukemia (AML) (Belson, Kingsley et al. 2007, Jin, Xu et al. 2016). Potential exposure of children to ionizing radiation may occur during the gestational phase or in the postnatal period (Belson, Kingsley et al. 2007). Other risk factors already accepted includes prenatal x-ray exposure; postnatal exposure to high doses of radiation and very specific genetic conditions, such as Down Syndrome, among other syndromes (INCA 2018).

In a study associating haplotypes with patients that were exposure to radiation, Chokkalingam, Bartley et al. (2011) observed that, when analyzing 32 genes responsible for cell cycle repair pathways, 4 haplotypes from *APEX1*, *BRCA2*, *RAD51* and *ERCC2* genes demonstrated a risk association. Also, they showed that 3 genes (*NBN*, *XRCC4* and *CDKN2A)* were associated with structural and numerical genetic alterations in ALL patients, showing genetic susceptibility regarding to ionizing radiation.

The hypotheses of population mixture and late infection suggest that a poor immune system in the early stages of human development may cause abnormal immune responses against infections, allowing an altered cell to develop. Both hypotheses are similar to the sanitary hygienic hypothesis, which explains the emergence of the original cell by the increased frequency of allergies during the first years of life. Many studies support the hypothesis of infections and the immune system as etiological factors of ALL (Greaves 2006, Chang, Wiemels et al. 2010, Han, Lan et al. 2010, Chang, Tsai et al. 2012, Wiemels 2012). However, little is known about the role of genes in this etiology.

Environmental factors have been evaluated as possible predisposing factors in the development of ALL but studies have produced conflicting results. Quiroz, Aldoss et al. (2019) describes chromosomal abnormalities and specific genomic landscape in Latinos with ALL and their association with unfavorable prognosis, focusing on the higher frequency of Philadelphia chromosome-like ALL. These authors compared the distribution of ALL throughout the various countries in Latin America in an attempt to shed some epidemiological light on the genetic ancestry of ALL.

Other environmental factor include hydrocarbons, especially benzene, pesticides, and the consumption of alcohol, cigarettes, and illicit drugs during pregnancy, which have been described as predisposing factors for childhood ALL (Infante-Rivard, Labuda et al. 1999,

Hashibe, Straif et al. 2005, Metayer, Zhang et al. 2013, Chunxia, Meifang et al. 2019). Some biological agents, such as viruses, as the Epstein Barr virus (EBV) and the human T-cell lymphotropic virus 1 (HTLV-1), also remains as risk factors for ALL. These risk factors may disrupt the immune response or even absence of contact with microorganisms during childhood (Greaves 2006, Inaba, Greaves et al. 2013).

The relationship between the immune system and ALL is a complex process involving the interaction of many cells including leukocytes, epithelial barriers, complement proteins, cytokines, T helper 1 (Th1), T helper 2 (Th2) cells, Regulatory T (Tregs) and auxiliary T 17 (Th17), in addition to molecules differentiation cluster 28, receptor for CD80 and CD86 (CD28), receptor gamma II for Fc portion of immunoglobulins G (FCGR2), transcription factor T-cell-specific trans-activator (GATA3), signal transducer and transcription activator protein 4 (STAT4), STAT6 and many others (Chang, Wiemels et al. 2010). Variations in the genes of these cells can affect the development and function of immune responses, and thus increase susceptibility to ALL (Han, Guo et al. 2013).

Many epidemiological studies aimed to identify risk factors in the etiopathogenesis of ALL, have been conducted in North America or European countries, where the exposure pattern for infectious diseases is not known to be like in the developing or tropical countries. It has been suggested that the higher prevalence of ALL in these countries is due to lower exposure to common mainly viral childhood infections and also to lower social contact between children, which would lead to an ineffective or altered immune response in the presence of a late common infections. This hypothesis is particularly associated with common ALL ($CD10^{+}$), which is the most prevalent leukemia in younger children. Although it is not a causal factor, it could be a second determinant step in leukemogenesis. In developed countries with high standards of hygiene, there is already a discussion about the immune profile acting on the increased incidence of certain serious and emerging childhood diseases, such as diabetes, asthma and ALL (Yazdanbakhsh, Kremsner et al. 2002).

There is the evidence that advanced parental age is associated with increased childhood ALL risk and this association was most marked among children aged 1-5 years. Employing datasets with cytogenetic information may further elucidate involvement of each parental component and clarify underlying mechanisms (Petridou, Georgakis et al. 2018).

Study by Orsi, Magnani et al. (2018) included 7,847 ALL cases and 11,667 controls aged 1 to 14 years. Data were obtained using standardized questionnaires. Pooled odds ratios (ORs) and 95% confidence intervals (CIs) were estimated by unconditional logistic regression adjusted for age, sex, study, maternal education and maternal age. Contact with cattle in the

first year of life was inversely associated with ALL (OR = 0.65, 95% CI: 0.50, 0.85). Inverse associations were also observed in contact with dogs (OR = 0.92, 95% CI: 0.86, 0.99) and cats (OR = 0.87, 95% CI: 0.80, 0.94) in the first year of life. However, there was no evidence of significant association with agricultural residence in the first year.

In this context, there is a concern in the developing country, with a broad heterogeneous territorial population exposed to different infectious agents and hygiene levels, and also a concern in children, that, in addition to being exposed to common infectious diseases in this age group, are prone to the development of regional diseases, such as parasitic diseases, mainly due to climatic and sanitation differences.

Although the recognition of leukemia among childhood diseases is well established, as well as the role of treatment groups for this disease, very little is known about the involvement of the environment in the development of the disease. As pointed out in this review, true biological profile of childhood leukemia is unknown, requiring in-depth studies regarding different geographical regions, and exposure to risk factors, such as exposure to ionizing rays, xenobiotics and chemical, in addition to the presence of infectious microorganisms.

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4. 2 Artigo 2

Association between NAD(P)H quinone oxidoreductase 1 (*NQO1*) gene polymorphism and acute lymphoblastic leukemia prognostic factor

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Abstract

Acute lymphoblastic leukemia (ALL) is a heterogeneous disease, regarding genetic changes, treatment response and prognosis. Although overall survival of childhood ALL patients after chemotherapy has improved over the years, reaching 80% cure rates, an attempt to improve diagnosis and efficacy of current therapeutic protocols has been proposed. Quinones are a class of organic compounds derived from aromatic hydrocarbons, found in various biological systems. Involved in the metabolism of quinones, the enzyme NAD(P)H quinone oxidoreductase 1 (NQO1) is a reductase that protects against the toxic effects of anticancer drugs. The *NQO1* gene has a single base genetic polymorphism rs1800566 (C609T) that leads to a low enzymatic activity. As the enzyme encoded by this gene carries out an important step in cellular homeostasis, it is clear that the absence or decrease of its activities may lead to an increase in toxicity and susceptibility to the development of malignant neoplasms, such as ALL. Within this context, this work aimed to analyze the polymorphism in the *NQO1* gene in ALL patients and neoplastic free controls, in the search for markers related to susceptibility, progression and therapeutic response. Peripheral blood samples from 74 patients with confirmed diagnosis and from 115 controls, had their genomic DNAs extracted and amplified by methodology based on the polymerase chain reaction and subsequent restriction fragment length polymorphism (PCR-RFLP). The case-control study did not indicate an association between the presence of the polymorphic variants and the susceptibility to ALL or survival. However, the C609T polymorphism correlated with relapse in ALL (p=0.03). Thus, in the sample of this work, the *NQO1* gene was shown as a possible candidate for molecular marker of prognosis or progression for the oncogenesis of ALL.

Keywords: ALL, genetic, NQO1, prognosis, survival, relapse, treatment.

INTRODUCTION

It is known that oxidative stress resulting from excess reactive oxygen species and / or deficiencies in cellular antioxidant capacity may play an important role in the etiology of cancer (Hong, Ambrosone et al. 2007). Thus, a balance between endogenous oxidants and antioxidants is commonly affected by the variation in genes involved in the generation and removal of reactive species (Tamimi, Hankinson et al. 2004, Kisaoglu, Borekci et al. 2013).

Quinones are a class of organic compounds derived from aromatic hydrocarbons, found in many biological systems, being part of the electron transport chain (Monks, Hanzlik et al. 1992, Monks and Jones 2002). Some quinones are used in the systemic treatment of cancer, such as anthracyclines (doxorubicin) and mitomycin C (Asche 2005). Involved in the metabolism of quinones, the enzyme NAD (P) H: quinoneoxidoreductase 1 (NQO1) is a

mandatory two-electron reductase that participates in the protection against the toxic effects of anticancer drugs, activates certain antitumor quinones (Ross, Kepa et al. 2000) and is involved in defense against reactive forms of oxygen (Nioi and Hayes 2004).

The gene encoding this enzyme (*NQO1*) has a single base genetic polymorphism (C609T) (rs1800566) located in exon 6, which causes a change in the amino acid sequence (Pro187Ser) (Traver, Horikoshi et al. 1992, Traver, Siegel et al. 1997). This mutation causes loss of function, where heterozygous individuals (CT) have low enzyme activity, whereas recessive homozygotes (TT) do not demonstrate their cytoprotective effects (Lanciotti, Dufour et al. 2005). This polymorphism has been associated with the risk of developing various types of cancer (Zhang, Schulz et al. 2003, Malik, Zargar et al. 2011, Yuan, Xu et al. 2011).

It is known that ALL is the most common malignancy in childhood. Regarding the known prognostic factors, they mainly include clinical and biological characteristics that are evaluated at the time of diagnosis, as well as the early response to treatment (Pui, Evans et al. 2008, Stanulla and Schrappe 2009).

ALL has been associated with reduced levels of NQO1 and sub expressed *NQO1* was found in leukemia cell line containing the heterozygous *NQO1* polymorphism (Wu, Oraee et al. 2015).

In general, there is a need to expand studies involving molecular markers responsible for different stages of the metabolism of xenobiotics, drugs and endogenous compounds. And also, the evaluation of the presence of these mutations in relation to prognostic parameters, progression and therapeutic response, is scarce in the world literature. Based on the above, in this work, we aimed the analysis of *NQO1* C609T genetic variants involved in endogenous and exogenous metabolism, and its possible involvement in ALL susceptibility and prognosis.

Material and Methods

Experimental Design

In the present study, a case-control study was conducted to compare the presence of the proposed polymorphism between the two groups, in the search for polymorphism associated with a susceptibility to the development of ALL. At the same time, a cross-sectional study was carried out to evaluate the association between the presence of polymorphisms and the prognostic factors of the patients.

Sample characterization

The study protocol was reviewed and approved by the human ethics committee of Londrina State University (CAAE N°. 17123113.4.0000.5231). Written informed consent was obtained from all study subjects (children's parents). Peripheral blood (5 mL) was collected from 74 patients (41 males and 33 females with ALL diagnosis, attended at the Londrina Cancer Hospital (LCH) and University Hospital (UH) of Londrina State University, Paraná, Brazil.

The diagnosis of ALL was classified according to the European Group for Immunophenotyping of Leukemias (EGIL) recommendations (Bene, Castoldi et al. 1995). Philadelphia chromosome status was assessed by cytogenetic analysis and by molecular methods, when applicable. Patients identified as having relapsed were selected and critical data (date of relapse, date of second remission if attained, date of subsequent relapse, survival status, date of last contact or death and cause of death).

For control group, blood samples of 115 individuals (40 males and 75 females) without cancer or inflammatory diseases (based on hematological, biochemical, and serological tests) were collected in UH and included as a control group.

Clinical characteristics

The protocol used by LCH was defined by the Brazilian Childhood Leukemia Treatment Group - GBTLI-LLA 99, updated on December 12, 2001 (Brandalise, Pinheiro et al. 2010). This protocol uses the classification of the National Cancer Institute and the *in vivo* therapeutic response evaluated at the 7th, 14th, and 28th days of induction treatment with four drugs (dexamethasone, vincristine, daunorubicin and asparaginase) and defines patients as high and low risk (GBTLI-LLA 2000). In the low-risk group, patients older than or equal to 1 year and less than 9 years old are included, leucometry less than 50,000/mm3 at diagnosis and less than 5,000/mm3 at the 7th day of treatment, absence of peripheral blasts and low bone marrow (BM) involvement on the 14th day of treatment and BM with low count of leukemic cells at 28th of induction, if there is infiltration of blasts in the central nervous system (CNS) at diagnosis, these should be absent on the examination of cerebrospinal fluid (CSF) on the 14th day of treatment. For the high-risk group, it is considered age less than 1 year and greater than or equal to 9 years, leucometry greater than 50,000/mm3 at diagnosis and greater than or equal to 5,000/mm3 on the 7th day of treatment, presence of leukemic blasts in the peripheral blood or extensive involvement of BM on the 14th day, evidence of extra-medullary leukemic involvement at the end of induction (Cazé, Bueno et al. 2010).

DNA Extraction

Genomic DNA was extracted from total peripheral blood by the Mini Spin Plus Extraction Kit (BioPur, Curitiba, Paraná, Brazil) according to the manufacturer's instructions. DNA samples were quantified by spectrophotometry on NanoDrop 2000c Spectrophotometer (ThermoScientific, Wilmington, Delaware, USA) at a wavelength of 260 nm.

Analysis of rs1800566 polymorphism of the *NQO1*

Genotyping was performed by polymerase chain reaction (PCR) and subsequent restriction fragment length polymorphism (RFLP) analysis. PCR was performed in final volume of 12.5 µl containing 100ng of DNA as standard, 1.25µl of 10X buffer, 1 unit of Taq-DNA polymerase (Invitrogen^{TM}, Carlsbad, USA), 100µmol of dNTPs and 10µmol of each primer (forward primer: 5' AAGCCCAGACCAACTTCT 3' and reverse primer 5' ATTTGAATTCGGGCGTCTGCTG 3'). The amplification of a 174pb fragment was evaluated by electrophoresis in 10% acrilamide gel stained with silver nitrate. The PCR products were enzymatically digested with 1 unit of HinfI enzyme (Boehringer, Mannheim, Germany) and visualized electrophoresis as described previously. The prevalent allele demonstrated a 174bp product resistant to enzymatic digestion, while the rare allele T (polymorphic) was recognized by the digestion enzyme, producing two fragments, one of 119bp and one of 55bp.

Statistical analysis

The case-control study for ALL susceptibility was assessed using statistical program GraphPadPrism version 6.00 for Windows (GraphPad Software, La Jolla California USA), through Odds ratio (OR), adopting an estimative of the relative risk at 95% confidence intervals (CI). Genotypic, dominant and recessive models were tested. Correlation analysis with ALL clinical outcome was analyzed by Spearman's Rho test using SPSS Statistics 22.0 software (SPSS Inc., Chicago, Illinois, USA). A p value < 0.05 was considered statistically significant.

RESULTS

The mean initial leucometry of treatment peripheral blood test revealed a white blood cell count of 40.830 leucocytes/mm3 with 50% blast cells. In the D7 (day 7 of induction) mean white blood cell count of 5.489 leucocytes/mm3 with 2,13% blast cells.

This study involved 74 ALL children patients, 41 males and 33 females (mean age 8.3 ± 2.3 years) with ALL diagnosis and 115 children, 40 males and 75 females (mean age 9.81 ± 1.7 years) for control group. All children analyzed in the study are from southern Brazil. Control

and ALL samples were tested for the Hardy Weinberg equilibrium and no deviation from expected genotype distributions were found ($p > 0.05$).

The case-control association study was performed to assess possible influences of (C609T) (rs1800566) *NQO1* gene polymorphism in ALL patients. Table 1 shows the genotype distributions.

Table 1. Case control study association for *NQO1* polymorphism in ALL patients and free neoplasia controls.

NQO		**LLA**		**CONTROL**		**OR (CI95%)**	**p value**
		N	**%**	**N**	**%**		
Genotypes	CC	42	(56.76)	44	(36.66)	1.00 (reference)	-
	CT	27	(36.48)	32	(39.02)	0.88 (0.46-1.72)	0,74
	TT	5	(6.76)	6	(7.32)	0.87 (0.25-3.08)	1,00
Recessive	CC+CT	69	(93.24)	76	(92.68)	1.00 (reference)	
	TT	5	(6.76)	6	(7.32)	0.99 (0.91-1.08)	1.00
Dominant	CC	42	(56.76)	44	(53.66)	1.00 (reference)	
	CT+TT	32	(43.24)	38	(46.34)	0.42 (0.40-1.45)	0.76
Allele Frequency	C	111	(75.0)	120	(73.17)	0.83 (0.50-1.39)	0.52
	T	37	(25.0)	44	(26.83)		

Recessive model: TT versus CT and CC; Dominant model: CT and TT versus CC

Regarding C609T polymorphism, ALL clinical presentation was evaluated. For these analyses, all clinicopathological parameters were categorized as shown in Table 2. Twenty-four patients were classified as low-risk and 50 as high-risk of relapse.

Table 2. Clinicopathological parameters analysis in relation to *NQO1* polymorphism in ALL patients.

		***NQO* genotypes** p (Spearman's Rho)			**Model** p (Spearman's Rho)
		CC n (%)	CT n (%)	TT n (%)	CT+TT n (%)
ALL classification	B	30 (40.54)	26 (35.15)	4 (5.40)	30 (93.75)
	T	12 (16.21)	1 (1.35)	1 (1.35)	2 (6.25)
		p= 0,03* (-0,26)			p= 0.04* (-0.24)
Gender	Male	24 (32.42)	14 (18.92)	3 (4.06)	17 (53.13)
	Female	18 (24.33)	13 (17.56)	2 (2.71)	15 (46.87)
		p=0.79 (0.31)			p=0.74 (-0.40)
Risk	Low	11 (14.86)	12 (16.21)	1 (1.35)	13 (40.63)
	High	31 (41.89)	15 (20.27)	4 (5.42)	19 (59.37)
		p=0.66 (0.53)			p=0.19 (0.15)
Relapse	Absent	30 (40.54)	24 (32.43)	5 (6.76)	29 (90.63)
	Present	12 (16.21)	3 (4.05)	0	3 (9.37)
		p=0.03* (-0.25)			p=0.04* (- 0.24)
	Cure	30 (40.54)	24 (32.43)	5(6.76)	29 (87.88)
	Death	11 (14.86)	4 (5.41)	0	4 (12.12)
		p=0.77 (-0.21)			p=0.15 (0.17)

* $p<0.05$

DISCUSSION

The collected evidence by Brisson, Alves et al. (2015) suggests that genetic polymorphisms in some metabolic genes are capable of modulating the risk of leukemia, especially when associated with environmental exposures, including *NQO1* polymorphism. In the present study no association was verified with *NQO1* C609T polymorphism (Table 1).

ALL and chronic myelogenous leukemia are associated with reduced levels of NQO1 and sub expressed *NQO1* was found in human HL-60 (leukemia cell line containing the heterozygous polymorphism *NQO1*) (Wu, Oraee et al. 2015).

A meta-analysis is generally acknowledged as one of the best methods for secondary research, and so it was applied in the study from He, Zhai et al. (2017) with the aim of elucidating how the *NQO1* C609T polymorphisms are related to the risk of ALL. This meta-analysis included 28 relevant studies involving 5,953 patients and 8,667 controls. There were increased risks of ALL in all subjects for carriers of the *NQO1* C609T polymorphism. T allele carriers might be more susceptible and thus should pay more attention to avoid environmental and lifestyle risk factors.

It is known that NQO1 protein protects cells against oxidative stress induced by quinine. The homozygosity of this polymorphism is associated with the loss of enzymatic activity due to the instability of the protein product (Ouerhani, Cherif et al. 2013). Already the heterozygous genotype showed a three-fold decrease in enzymatic activity compared to the wild-type allele (Zheng, Wang et al. 2014). NQO1 has been described as an anticancer enzyme since it plays a protective role in carcinogenesis by modifying internal exposure to bioactivated carcinogens (Li and Zhou 2014) and, in the case of a defective enzyme, may play a role in carcinogenesis.

A total of 34 studies including 12,043 digestive tract cancer cases and 15,209 healthy controls were included in the meta-analysis from Yadav, Kumar et al. (2018). Stratified analysis based on ethnicity indicated a significant association between *NQO1* C609T polymorphism and digestive tract cancer risk in the Asian as well as in Caucasian populations suggesting that the *NQO1* C609T polymorphism is a risk factor for digestive tract cancers, including gastric cancer and colorectal cancer.

Study from Fan, Hu et al. (2014) firstly showed that individuals carrying *NQO1* C609T variant allele are more susceptible to hepatocellular carcinoma, particularly for Asians. In this context Hu, Hu et al. (2014), through meta-analysis involving 4,000 subjects, reported that the *NQO1* gene C609T polymorphism increases the risk for gastric cancer, especially in Asian populations. Therefore, this polymorphism in a way has been involved with ethnicity for association to disease risk.

Drug metabolizing enzymes are responsible for the metabolism of various chemotherapeutic agents and many of these enzymes are genetically polymorphic. These polymorphisms may influence the enzymatic activity and could improve the response to the treatments, reducing the risk of relapses.

Since the enzyme NQO1 is related to the metabolism of antitumor drugs and the allelic variant rs1800566 modifies its activity, it was verified in a study relating changes in the response to adjuvant therapy with doxorubicin and cyclophosphamide, with or without tamoxifen, that the polymorphic genotype TT was associated with poor clinical outcome and low probability of treatment success, demonstrating a possible modulating effect of this polymorphism on the efficacy of adjuvant breast cancer therapy (Jamieson, Cresti et al. 2011).

Study from Megías, Montesinos et al. (2015) reveals that, as in other cancers, there is a prognostic impact of anthracycline metabolism gene polymorphisms in adult AML patients. Several associations were obtained including *NQO1* polymorphisms and toxicities.

Many authors have investigated the influence of polymorphisms in genes regarding metabolism to propose biomarkers in clinical practice in order to individualize chemotherapy schemes (Megias-Vericat et al., 2018; He et al., 2017).

In our work, the C609T polymorphism correlated with relapse in ALL, where the T carriers correlated negatively with relapse (Table 2, p=0.04). It appears that, although we have not found association with risk, genetic variants in metabolic enzymes such as *NQO1* may modulate the individual prognosis to relapse after chemotherapy.

ALL patients are known to be treated with drugs classified as quinones, such as anthracycline Daunorubicin. From this perspective, it is suggested that patients with the T allele for the *NQO1* C609T polymorphism may have a slower metabolism of the chemotherapeutic agent and therefore result in a longer time of action of the drug and providing a positive effect on anticancer therapy, with lower relapse rates.

CONFLICT OF INTERESTS: The authors declare that there are no conflicts of interest.

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4.3 Artigo 3

Possible involvement of glutathione S-transferase in pediatric patients with acute lymphoid leukemia

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Abstract

Leukemia is the most frequent form of cancer in children and adolescents under 14 years of age, accounting for about 31% of malignancies before 15 years of age, with a maximum incidence of 80 to 90 cases per million in the age group 2 to 3 years. Acute lymphoblastic leukemia (ALL) is a blood malignancy distinguished by an excessive buildup of lymphoid progenitor cells in blood and bone marrow. Better understanding of the mechanisms of progression of childhood leukemias and response to treatment could improve treatment response rates and increase patient survival. Drug metabolism and efflux are defense mechanisms responsible for protection against toxic agents and are involved in the biotransformation of various xenobiotics. These proteins are polymorphic, and these polymorphisms alter the enzymatic activity and can modify the response to the treatment and its resistance. The genes *GSTM1* and *GSTT1* encode phase II enzymes of metabolism, responsible for conjugation with other substances to facilitate excretion. *GSTM1* and *GSTT1* genes have a polymorphism that causes homozygous deletion of the gene. These polymorphisms lead to altered enzymatic activity. Thus, the present study evaluated the influence of *GSTT1* and *GSTM1* polymorphisms and plasma levels of GST on the risk of progression and response to treatment in 64 pediatric patients with ALL and 68 controls. Both polymorphisms were not associated with ALL susceptibility or risk of relapse and their different genotypes did not alter GST plasma levels. However, it was verified increase of GST concentration in ALL compared to control group ($p<0.0001$), however, no association of GST plasma levels with the analyzed parameters was found. This work has shown increased plasma GST levels of patients with ALL suggesting possible involvement of this protein in treatment.

Keywords Acute lymphoblastic leukemia, Genetic polymorphisms, *GSTM1*, *GSTT1*.

Introduction

Acute lymphoid leukemia (ALL) is a malignant disorder that originates from a single hematopoietic precursor affecting the B or T cell line. The acquisition of a series of genetic alterations interrupts the normal processes of maturation, leading to the blockage of differentiation and proliferation of the transformed cell (Graux 2011).

Like cancer in general, ALL probably originates from interactions between exogenous and endogenous exposure and genetic susceptibility (Inaba, Greaves et al. 2013).

The pathophysiology of ALL involves complex mechanisms where genetic alterations and environmental factors may be related. However, some of the key points in ALL pathophysiology are its monoclonal origin, the uncontrolled cell proliferation by stimulation of growth factor receptors, the lack of response to inhibitory signals and cell longevity conditioned by decreased apoptosis (Arreola, Puebla-Pérez et al. 2013).

Glutathione S transferase (GSTs) belongs to the super family of phase II dimeric metabolic enzymes, which plays an important role in the cell protection system. GSTs are a family of enzymes encoded by five gene classes μ, θ, π, α, σ implicated in the detoxification of a wide range of compounds, including xenobiotics, pesticides, environmental carcinogens, PAHs and some chemotherapeutic drugs (including alkylating agents, doxorubicin and vincristein). Functional polymorphisms have been reported in at least three of the genes encoding GSTs, including *GSTM1*, *GSTT1*, and *GSTP*. *GSTT1* and *GSTM1* genes exhibited a higher degree of polymorphism, one of them being the complete deletion of the gene that causes the loss of enzymatic activity (Alves, Amorim et al. 2002).

The *GST* gene family can modulate leukemia risk through two potential mechanisms, mediating specific leukemogen metabolism or directly affecting redox potential in the cell, protecting DNA against free radical-induced damage (Dunna, Vure et al. 2013).

GST gene polymorphisms have been found to be associated with susceptibility to nonmalignant and malignant diseases, including acute myeloid leukemia (AML) (Alves, Amorim et al. 2002). The *GSTM1* gene, located on chromosome 1, is polymorphic having 3 variants, two of which are functional alleles (*GSTM1 A* and *GSTM1 B*), which have the same detoxification efficacy, and one deletion-null allele (*GSTM1 0*) (Widersten, Pearson et al. 1991). The *GSTT1* gene, located on chromosome 22, is also polymorphic and may have a null deletion phenotype. The homozygous deletion of the *GSTM1* gene is observed at frequencies ranging from 20 to 70% in different populations, while for the *GSTT1* gene this variation is from 11 to 38% (Arruda, Grignolli et al. 1998).

The enzymes encoded by these genes perform an important step in detoxifying xenobiotics, it is clear that the absence of their activity may lead to increased toxicity and susceptibility to the development of malignant tumors (Deakin, Elder et al. 1996, Cascorbi 2006). Several studies have detected a high frequency of null genotype for these two genes in individuals with different cancers, especially lung (Hirvonen, Husgafvel-Pursiainen et al. 1993), bladder (Johns and Houlston 2000) and oral cavity cancer (Sato, Sato et al. 1999).

Patients with a null *GST* genotype were believed to exhibit impaired detoxification of environmental genotoxic agents and chemotherapeutic drugs, leading to an increased risk of developing primary and secondary cancers and treatment-related complications. Children with the null *GSTM1* genotype have been reported to be at increased risk of developing ALL (Krajinovic, Labuda et al. 1999, Saadat and Saadat 2000). Crump, Chen et al. (2000) reported no association between *GSTT1*, *GSTM1* and AML gene deletions. Patients with secondary AML had a slightly higher prevalence of deletions in the *GSTT1* and *GSTM1* genes compared

to patients with new AML. The null *GSTM1* genotype was found to correlate with an increased risk of these malignancy (Alves, Amorim et al. 2002).

In this context, the propose of this work was to investigate *GSTM1* and *GSTT1* genetic polymorphisms and plasma glutathione level in pediatric patients with ALL.

Material and Methods

Individuals

The study was approved by Human Ethics Committee of the Londrina State University, Londrina, Parana, Brazil (CAAE N° 17123113.4.0000.5231). Informed consent was obtained from all individuals (or their parents) included in the study. Peripheral blood (5 mL) was collected from 64 patients (36 males and 28 females) with ALL diagnosis attended in the Londrina Cancer Hospital (LCH) and University Hospital (UH) of Londrina State University, Paraná, Brazil.

The diagnosis of ALL was classified according to the European Group for Immunophenotyping of Leukemias (EGIL) recommendations (Bene, Castoldi et al. 1995). For control group, blood samples of 68 children (32 males and 36 females) without cancer or inflammatory diseases (based on hematological, biochemical and serological tests) were collected and included as a control group. Age ranging of people involved in this study was from 3 months to 19 years old.

Analysis of *GSTM1* and *GSTT1* polymorphisms

Co-amplification reaction was carried out based on multiplex polymerase chain reaction (PCR) protocol proposed by Abdel-Rahman, El-Zein et al. (1996) with modifications, under the following conditions: enzyme buffer (20mM Tris-HCl pH 8.4; 50mM KCl); 2mM $MgCl_2$; 10 pmol of each primer; 1.25 U Taq DNA polymerase; 2 mM dNTPs; 4ng/µL of genomic DNA and ultrapure sterile water to complete the final volume of 25µL.

Primers for *CYP1A1* gene amplified a non-polymorphic 312 base-pairs (bp) fragment, used as an internal PCR control. Fragments of 215 and 480 bp were observed, respectively, in the *GSTM1* and *GSTT1* positive individuals. The absence of *GSTM1* or *GSTT1* amplification, in the presence of internal control, indicated the respective null genotypes.

Fragments were amplified, submitted to electrophoresis in 10% polyacrylamide gel and visualized by silver nitrate staining.

Plasma Glutathione quantification

100 µL aliquots of plasma were deproteinized with 100 µL of 50% trichloroacetic acid and centrifuged for 5 minutes at 4000 revolutions per minute. A 50 µL volume of this supernatant was added to the microplate and mixed with 200 µL Tris-HCl buffer solution (0.4 molar, pH 8.9). To control the reaction, a standard curve with serial dilution of the 0.03 M GSH solution was made. Finally, 50 µL of 0.01 M 5,5′-ditiobis (2-nitrobenzoic acid) (DTNB) (prepared in methanol) was added to all samples and to the curve. One detection was performed by 405 nm wavelength spectrophotometry.

Statistical analysis

The association study for risk of ALL development and clinical outcome was calculated by Odds ratio (OR) at 95% confidence interval (CI) using statistical program Graph Pad Prism version 6.00 for Windows (GraphPad Software, La Jolla California USA). Correlation analysis with ALL clinical outcome was analyzed by Tau_b Kendall test using SPSS Statistics 22.0 software (SPSS Inc., Chicago, Illinois, USA). A p value < 0.05 was considered statistically significant.

Results

In the study, the average age from ALL patients and controls were 8.4 and 9.5 years old, respectively. The mean initial leucometry of treatment peripheral blood test revealed a white blood cell count of 44.173 leucocytes/mm3 with 52.43% blast cells. In the D7 (day 7 of induction) mean white blood cell count of 3.885 leucocytes/mm3 with 2.5% blast cells.

Electrophoretic profile of *GSTM1* and *GSTT1* polymorphism is represented in Figure 1.

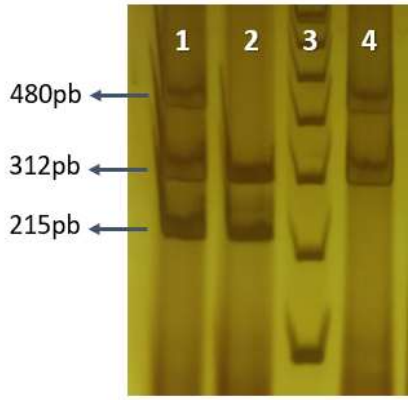

Figure 1. PCR profiles for *GSTM*1 and *GSTT1* gene polymorphisms. 1: positive genotypes; 2: *GSTM1* positive; 3: Ladder 100 bp; 4: *GSTT1* positive.

Genetic variants involved in exogenous and endogenous metabolism were analyzed in ALL patients and controls (Table 1). The double deletion (*GSTM1* null + *GSTT1* null) genotype was found only one ALL patient, but not in controls.

Table 1. Genotypic distribution and association study of *GSTT1* and *GSTM1* polymorphisms between ALL patients and neoplastic free controls.

	GSTT1 Presence	*GSTT1* Deletion	*GSTM1* Presence	*GSTM1* Deletion
ALL	52 (81.25%)	12 (18.75%)	34 (53.13%)	30 (10.87%)
Control	54 (84.37%)	14 (15.63%)	42 (61.76%)	26 (38.24%)
OR	1.12		0.70	
CI (95%)	0.47 – 2.65		0.35 – 1.43	

Odds Ratios (OR), confidence intervals (CI 95%)

Kendall's tau-b test was performed to test for associations between *GSTT1* and *GSTM1* polymorphisms and clinicopathological parameters. *GSTM1* and *GSTT1* deletion was not associated with clinicopathological parameters (Table 2).

Table 2. Association analyses between *GSTT1* and *GSTM1* polymorphisms and ALL clinicopathological parameters

Parameter	***GSTT1***		***GSTM1***	
	WT	**Del**	**WT**	**Del**
ALL Classification				
B	44 (69%)	9 (14%)	29 (45%)	24(38%)
T	8 (12,5%)	3 (4,5%)	5 (8%)	6 (9%)
p	0.42		0.74	
Risk				
Low	17 (27%)	2 (3%)	9 (14%)	10 (15%)
High	35 (55%)	10 (15%)	25 (40%)	20 (31%)
p	0.48		0.59	
Relapse				
Absent	39 (61%)	11 (17%)	29 (45%)	21 (33%)
Present	13 (20%)	1 (2%)	5 (8%)	9 (14%)
p	0.27		0.22	
Status				
Cure	41 (64%)	10 (16%)	30 (47%)	21 (33%)
Death	11 (17%)	2 (3%)	4 (6%)	9 (14%)
p	1.00		0.12	

Fisher's exact test, $p<0.05$

Glutathione level in ALL and healthy donors are shown in Figure 2. The plasma level of healthy donors presented mean concentration of 12.16 nmol/L, while in plasma of patients showed higher concentration, 17.41 nmol/L ($p < 0.0001$)

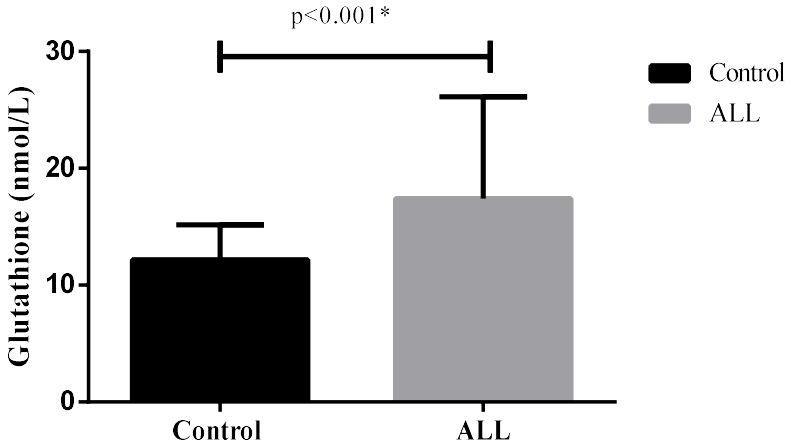

Figure 2. Glutathione plasma levels. The results were expressed in nmol/L for GST. Each sample including controls (CC) and ALL patients was assayed in two biological replicates. S.E.M. as error bars.

In the present study, there was no significant difference between glutathione levels in relation to *GST* genotypes. Glutathione level in ALL and *GSTT1* and *GSTM1* polymorphisms are shown in Figure 3. The plasma level of patients with *GSTT1* wt: 17.64 nmol/L; *GSTT1* del: 18.71 nmol/L and for *GSTM1* wt:17.92 nmol/L and *GSTM1* del :17.72 nmol/L (Figure 3).

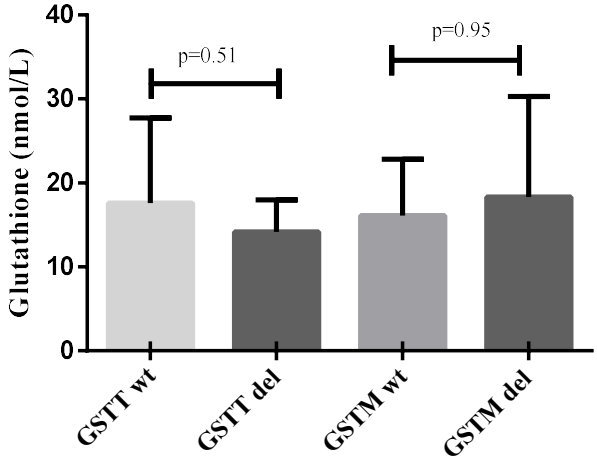

Figure 3. Glutathione plasma levels and *GST* polymorphisms. The results were expressed in nmol/L for GST. Each sample including controls (CC) and ALL patients was assayed in two biological replicates. S.E.M. as error bars.

Tau-b Kendall tests were performed to test for associations glutathione and clinicopathological parameters and there was no significant difference between glutathione levels in relation to clinicopathological parameters (Table 3).

Table 3. Association analyses between *GSTT1 and GSTM1* polymorphisms and ALL clinicopathological parameters

Prognostic Parameters		Number of patients	GST (mean nmol/L)	p value	τ* value
ALL Classification	B	53 (83%)	17.64	0.663	-0.050
	T	11 (17%)	17.90		
Risk	Low	19 (30%)	18.41	0.522	-0.074
	High	45 (70%	17.73		
Relapse	Absent	50 (78%)	17.64	0.991	-0.001
	Present	14 (22%)	17.79		
Status	Cure	51 (80%)	17.64	0.633	-0.055
	Death	13 (20%)	17.14		

* Kendall's tau-b test (τ), $p<0.05$ values were considered statistically significant.

Discussion

The present study found no association of *GSTM1* and *GSTT1* genetic polymorphisms in childhood ALL but showed increased of plasma GST in ALL patients. However, Weich, Nunez et al. (2015) conducted a study in 36 patients and 133 healthy individuals and verified that the *GSTM1*-null genotype was associated with a lower risk of developing acute leukemia (p=0.013; OR: 0.31; CI: 0.12-0.80) no differences were observed for *GSTT1* gene.

Moulik, Parveen et al. (2014) evaluated association among north Indian ALL children and conducted case-control study and meta-analysis. The genotypes in 100 ALL children and 300 healthy controls were compared where both *GSTM1* (OR 1.54, 95%CI 1.12-2.10) and *GSTT1* (OR 1.63, 95%CI 1.32-1.99) null genotypes were associated with increased risk in Asian subjects.

Dunna, Vure et al. (2013) developed a study including 152 of ALL and 251 control samples and analyzed *GSTM1* and *GSTT1* polymorphisms through multiplex PCR method. It was verified that absence of both *GSTM1* and *GSTT1* might confer increased risk of developing ALL.

Zhang, Zhang et al. (2017) analyze the relationship between *GST* polymorphism and ALL risk involving 27 studies which included 3736 cases of ALL and 5549 controls and concluded that *GSTM1* and *GSTT1* polymorphisms may be correlated with an increased risk of ALL. In this context is conceivable that *GSTM1* and *GSTT1* null genotypes may thus play a role in leukemogenesis. Li, Zhu et al. (2017) related that *GSTM1* null genotype deletion may play a role in risk of leukemia. However, a meta-analysis from Zhao, Ma et al. (2018) suggested significant association with *GSTM1* variants and the risk of childhood ALL while no association were found for *GSTT1* variants.

In the other meta-analysis of 16 published studies performed by Xu and Cao (2014), 16 published studies showed that *GSTT1* null variant was significantly associated with risk of childhood ALL. However, in subgroup populational analysis showed that *GSTT1* null variant was significantly associated with risk of Asian childhood ALL and no association in both Caucasians and Africans.

Some results reveal that homozygous null genotypes of *GSTM1* and *GSTT1* does not influence ALL susceptibility among adult patients (Zehra, Zehra et al. 2018). The study from Guven, Unal et al. (2015) in Turkish population demonstrated no difference in the prevalence of the *GSTM1* and *GSTT1* null genotypes between the childhood ALL patients and the controls. In another study, Zareifar, Monabati et al. (2013) reported that there was no statistically significant relationship between *GSTT1* and *GSTM1* mutations or between double null status, prognostic factors and relapse in ALL.

In a systematic review and meta-analysis of 30 published case-control studies realized by Ye and Song (2005) was suggest that *GSTM1* and *GSTT1* polymorphisms appear to be associated with a modest increase in the risk of ALL. It is conceivable that *GSTM1* and *GSTT1* null genotypes may thus play a role in leukemogenesis.

Although association between *GSTM1, GSTT1* and risk of childhood ALL remains controversial, it is plausible to note that in studies with association between *GST* and ALL genetic polymorphisms, ODs values are well as low-value confidence intervals. In addition, the population presenting this association are predominantly Asian.

It is known that there is genetic mixture of the Brazilian population, characterized by an intense ethnic mixture. Most of the sample population of this study comes from the

southern region of Brazil, which is represented by ethnicity of Polish and European origin. It is reasonable to suppose that there is an ethnicity influence for these polymorphisms studied.

Glutathione is a thiol existing in eukaryotic cells in the reduced (GSH) and oxidized form (N Kaplowitz, T Y Aw et al. 1985). The reduced form is commonly found in the body with cell concentration levels from 5 to 10 millimoles (mM) (Wu, Fang et al. 2004) and integrates the process of xenobiotics metabolization facilitating the excretion of these compounds by the conjugation to these products through the GSTs (Oakley 2011). Thus, it was also analyzed a possible relationship between *GSTT1* and *GSTM1* polymorphisms in relation to glutathione plasma level, but no significance was found. However, our study evidenced the increased of glutathione plasma levels in ALL patients.

The action of these enzymes includes the interaction between their N-terminal residue with the thiol group of the glutathione peptide in its reduced form (γ-L-glutamyl-L-cysteine-glycine, GSH), providing the conjugation of compounds to be excreted, including carcinogens, drugs and products of metabolism (Dirr et al., 1994; Oakley, 2011). In this context GST increase may be due to patients undergoing ALL therapy.

It is worth mentioning some limitations of this work, such as the lack of analysis of other genes that regulate the detoxification phase I, as well as other *GST*, besides the reduced number of ALL patients.

Nonetheless, further molecular and functional investigations are necessary to determine whether the association of GST plasma levels may also be influenced by other polymorphisms.

Conflict of Interests: the authors declare that there are no conflicts of interest.

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5 CONCLUSÕES

Embora associações entre o polimorfismo genético rs1800566 (C609T) do gene *NQO1* não tenha sido encontrado quanto a suscetibilidade e a sobrevida dos pacientes com LLA, o presente trabalho verificou que os portadores do alelo T apresentaram menor recidiva.

Quanto aos polimorfismos genético *GSTM1* e *GSTT1* não houve associação quanto a suscetibilidade, o prognóstico de risco, a recidiva e a sobrevida dos pacientes com LLA.

O estudo não evidenciou associações entre os níveis plasmáticos de glutationa com os polimorfismos genéticos *GSTM1* e *GSTT1*, assim como não evidenciou associações entre os níveis plasmáticos de glutationa e a suscetibilidade com o prognóstico de risco, a recidiva e a sobrevida dos pacientes com LLA.

O presente trabalho demonstrou maior concentração dos níveis plasmáticos de glutationa nos pacientes com LLA em relação aos dos doadores saudáveis livres de neoplasias.

6 CONSIDERAÇÕES FINAIS

Há evidências crescentes de que a predisposição para LLA está associada à exposição a produtos químicos, como benzeno e agentes quimioterápicos. As enzimas envolvidas no metabolismo dessas substâncias cancerígenas têm sido estudadas como fatores de risco potenciais para o desenvolvimento da doença.

Esperamos que os resultados encontrados e os caminhos sugeridos neste trabalho contribuam para a expansão da compreensão dos aspectos clínicos, moleculares e terapêuticos da patogênese da LLA infantojuvenil, visando o benefício destes pacientes e de outras doenças com aspectos similares.

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ANEXOS

ANEXO A

Aprovação do comitê de ética em pesquisa envolvendo seres humanos da Universidade Estadual de Londrina

Universidade Estadual de Londrina

PARANÁ
GOVERNO DO ESTADO

COMITÊ DE ÉTICA EM PESQUISA ENVOLVENDO SERES HUMANOS
Universidade Estadual de Londrina
Registro CONEP 5231

Parecer CEP/UEL:	189/2013
CAAE:	17123113.4.0000.5231
Data da Relatoria:	30/09/2013
Pesquisador(a):	Maria Angelica Ehara Watanabe
Unidade/Órgão:	Programa de PG em Patologia Experimental

Prezado(a) Senhor(a):

O "**Comitê de Ética em Pesquisa Envolvendo Seres Humanos da Universidade Estadual de Londrina**" (Registro CONEP 5231) – de acordo com as orientações da Resolução 466/12 do Conselho Nacional de Saúde/MS e Resoluções Complementares, avaliou o projeto:

"Estudo de marcadores genéticos, epigenéticos, moleculares e imunológicos em câncer."

Situação do Projeto: **Aprovado**

Informamos que deverá ser comunicada, por escrito, qualquer modificação que ocorra no desenvolvimento da pesquisa, bem como deverá apresentar ao CEP/UEL, via Plataforma Brasil, relatório final da pesquisa.

Londrina, 30 de setembro de 2013.

Profa. Dra. Alexandrina Aparecida Maciel Cardelli
Coordenadora do Comitê de Ética em Pesquisa Envolvendo Seres Humanos
Universidade Estadual de Londrina

Comitê de Ética em Pesquisa Envolvendo Seres Humanos - UEL

Campus Universitário: Rodovia Celso Garcia Cid (PR 445), Km 380 - Fone (43) 3371-4000 - PABX - Fax 3328-4440 - Caixa Postal 6001 - CEP 86051-990 - Internet http://www.uel.br
LONDRINA - PARANÁ - BRASIL

ANEXO B

UNIVERSIDADE ETADUAL DE LONDRINA

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

Informações sobre a pesquisa:

Você está sendo convidada (o) a participar, como voluntária (o), da pesquisa intitulada **"Estudo de marcadores genéticos, epigenéticos, moleculares e imunológicos em câncer"**, que tem por objetivo analisar determinados tipos de moléculas que podem influenciar na imunidade da paciente. Você será esclarecida (o) sobre a pesquisa em qualquer aspecto que desejar. Sua participação não é obrigatória e, a qualquer momento, você poderá desistir de participar e retirar seu consentimento, sem que isso acarrete qualquer penalidade.

Procedimentos do Estudo:

Os procedimentos da pesquisa envolvem a obtenção de 5mL de sangue periférico para análise das células e moléculas do sistema imunológico.

Confidencialidade da Pesquisa

As informações obtidas através desta pesquisa serão confidenciais e asseguramos o sigilo sobre sua participação. Os dados não serão divulgados de forma a possibilitar sua identificação.

A amostra de sangue obtidos será utilizada para obtenção de DNA e RNA para a realização deste projeto. A participação no estudo não acarretará custos para você e não haverá nehuma compensação financeira adicional. A coordenadora do projeto é a Profª. Drª Maria Angelica Ehara Watanabe, que pode ser encontrada no endereço: Rod. Celso Garcia Cid, 445, Departamento de Ciências Patológicas, Centro de Ciências Biológicas, Universidade Estadual de Londrina, CEP: 86051-970, Tel / Fax: (43) 3371-5629, como também procurar o Comitê de Ética em Pesquisa Envolvendo Seres Humanos da Universidade Estadual de Londrina, na Avenida Robert Kock, nº 60, ou no telefone 3371 – 2490.

Pesquisador Responsável _____

RG::_____

Consentimento livre esclarecido e informado:

Eu, _____, RG _____, declaro que estou de acordo com as informações contidas neste documento, fui devidamente esclarecido pelo(s) pesquisador (es) dos objetivos e procedimentos da pesquisa de maneira clara e detalhada, e esclareci minhas dúvidas. Concordo em participar voluntariamente desse estudo permitindo a coleta do sangue do meu filho (a), sendo que poderei retirar meu consentimento a

qualquer momento, antes ou durante o mesmo, sem penalidades ou prejuízos no meu atendimento neste projeto.

Londrina, ____ de _____, 20 ___.

Assinatura do responsável (ou representante legal): _____