CARLOS EDUARDO CORAL DE OLIVEIRA

POLIMORFISMOS GENÉTICOS DO *FOXP3* E *CCR5*, E NÍVEIS PLASMÁTICOS DE TGFB1:

IMPLICAÇÃO NA PATOGÊNESE DA LEUCEMIA LINFOIDE AGUDA INFANTO-JUVENIL

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Tese apresentada ao curso de Pós-graduação em Patologia Experimental da Universidade Estadual de Londrina, como requisito parcial à obtenção do Título de Doutor em Patologia Experimental.

Orientadora: Profª. Drª Maria Angelica Ehara Watanabe.
Co-orientadora: Profª. Drª Roberta Losi Guembarovski.

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BANCA EXAMINADORA

Orientadora: Profª Drª Maria Angelica Ehara Watanabe
Universidade Estadual de Londrina - UEL

Profª Drª Aparecida de Lourdes Perim
Universidade Estadual de Londrina - UEL

Prof Dr Mario Augusto Ono
Universidade Estadual de Londrina - UEL

Profª Drª Karen Brajão de Oliveira Suzuki
Universidade Estadual de Londrina - UEL

Profª Drª Carolina Batista Ariza Tamarozzi
Universidade Estadual de Londrina - UEL

Londrina, 17 de dezembro de 2013.

Dedico esta tese às minhas duas
inspirações: a minha amada esposa
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OLIVEIRA, C.E.C **POLIMORFISMOS GENÉTICOS DO *FOXP3* E *CCR5*, E NÍVEIS PLASMÁTICOS DE TGFB1: IMPLICAÇÃO NA PATOGÊNESE DA LEUCEMIA LINFOIDE AGUDA INFANTO-JUVENIL**. 2013. 111 f. Tese (Doutorado em Patologia Experimental) - Universidade Estadual de Londrina. 2013.

RESUMO

A leucemia linfoide aguda (LLA) é a neoplasia maligna mais comum em crianças, se origina a partir de um precursor hematopoiético, acometido para a linhagem de células B ou T, que sofreu uma alteração genética, comprometendo os processos normais de maturação, com bloqueio da diferenciação e estímulo a proliferação da célula transformada. O acúmulo de mutações em genes, como aqueles do sistema imune e da regulação do ciclo celular, o desequilíbrio entre a diferenciação e a proliferação das células hematopoiéticas e os efeitos dos fatores de crescimento sobre a leucemogênese podem contribuir para o desenvolvimento e a evolução clínica da LLA. As quimiocinas e seus receptores desempenham funções cruciais nas respostas imunes e foram implicadas no desenvolvimento de diferentes doenças e processos patológicos, incluindo o câncer, particularmente regulando o processo de metástase. O receptor de quimiocinas CCR5 parece estar envolvido na patogênese do câncer, bem como um polimorfismo deletério em seu gene, denominado CCR5 delta32 foi associado ao risco de desenvolvimento de várias doenças. Além disso, o papel das células T regulatórias (Tregs) no câncer tem sido amplamente investigado. Envolvidas nos processos de tolerância e imunovigilância, as Tregs são caracterizadas pela expressão nominal do fator de transcrição FOXP3, e são induzidas pela presença de citocinas polarizadoras como o TGFB, IL-10 e IL-35. Tem sido demonstrada elevada presença de Tregs no sangue periférico de indivíduos com leucemia, embora os mecanismos que geram esta elevação não foram completamente elucidados. Ainda, a região promotora do gene *FOXP3* possui sequências sensíveis a fatores de transcrição ativados pela sinalização via TGFB, e pode apresentar polimorfismos genéticos, os quais foram associados a doenças autoimunes e alguns tipos de câncer. Neste contexto, o papel do receptor de quimiocinas CCR5 foi revisado na literatura mundial com vistas a avaliar as implicações deste receptor na imunidade em relação ao microambiente tumoral, sua relação com células T regulatórias, e o envolvimento do polimorfismo rs333 (CCR5 delta32) na susceptibilidade a LLA e seu valor prognóstico para a evolução de risco da doença, em um estudo de associação. O CCR5 deve exercer efeito indireto na progressão do câncer, pelo controle das respostas antitumorais, já que sua expressão parece promover o crescimento tumoral e contribuir para a metástase. Efeitos descritos de um antagonista para o CCR5 foram capazes de inibir o crescimento tumoral, o que sugere seu uso como um possível alvo terapêutico do câncer. Contudo, no estudo de associação caso-controle, o polimorfismo rs333 do *CCR5* apresentou distribuição semelhante de genótipos heterozigotos entre o grupo controle e leucêmico, demonstrando não haver implicações deste polimorfismo na susceptibilidade a LLA e na relação ao status de risco da doença. Adicionalmente, foram avaliados os polimorfismos rs3761548 e rs2232365 da região promotora do gene *FOXP3* entre pacientes com LLA e controles e o possível envolvimento no risco a LLA, inclusive na associação com a evolução clínica dos pacientes. As distribuições alélicas e genotípicas foram avaliadas e não houve diferenças significativas entre os grupos controle e leucêmico para os polimorfismos rs3761548 e rs2232365 do gene *FOXP3*. A presença dos alelos variantes e respectivos genótipos não foi associada à evolução clínica dos pacientes e, portanto, estes polimorfismos não foram considerados fatores de susceptibilidade genética para a LLA. Ainda, foi investigada a expressão proteica do fator de crescimento TGFB1, no plasma de

pacientes com LLA e controles livres de neoplasia, e buscou-se associar os níveis plasmáticos aos genótipos dos polimorfismos do gene *FOXP3*. Pacientes leucêmicos apresentaram níveis significativamente reduzidos de TGFB1 (p=0.0007), em comparação aos controles livres de neoplasia. Esta redução também foi observada na comparação entre os grupos controle e leucêmico de acordo com os genótipos dos polimorfismos rs3761548 e rs2232365 do gene *FOXP3*. Indivíduos portadores dos genótipos homozigotos variantes para os dois polimorfismos apresentaram níveis maiores de TGFB1 plasmático, embora diferença significativa tenha sido observada apenas para o polimorfismo rs3761548, no grupo controle. Estes resultados reforçam a hipótese da desregulação na via ativada pelo TGFB na LLA, e isto poderia exercer efeitos moduladores na expressão de FOXP3 nas células leucêmicas. Finalmente, o presente trabalho sugere que os polimorfismos genéticos da região promotora do *FOXP3* e a concentração plasmática do TGFB1 devem estar associados a patogênese da LLA.

Palavras-chave: CCR5. LLA. TGFB. FOXP3. Polimorfismos genéticos.

OLIVEIRA, C.E.C. **GENETIC POLYMORPHISMS OF *FOXP3* AND *CCR5*, AND TGFB1 PLASMA LEVELS: IMPLICATION IN THE PATHOGENESIS OF CHILDREN´S ACUTE LYMPHOBLASTIC LEUKEMIA**. 2013. 111 p. Thesis (PhD in Experimental Pathology) – State University of Londrina. 2013.

ABSTRACT

Acute lymphoblastic leukemia (ALL) is the most common malignant neoplasia in children, which originates from a hematopoietic precursor committed for B or T cell lineage that has undergone a genetic alteration, affecting the normal processes of maturation, disrupting cell differentiation and encouraging proliferation of transformed cell. The accumulation of mutations in genes, such as those of the immune system and cell cycle regulation, the imbalance between differentiation and proliferation of hematopoietic cells and the effects of growth factors on leukemogenesis may contribute to the development and clinical course of ALL. Chemokines and their receptors play crucial roles in immune responses and have been implicated in the development of different diseases and pathological processes, including cancer, particularly by regulating the process of metastasis. The chemokine receptor CCR5 appears to be involved in the pathogenesis of cancer, as well as a deleterious polymorphism in its gene, named CCR5 delta32 was associated with developing risk of several diseases. Furthermore, the role of regulatory T cells (Tregs) in cancer has been widely investigated. Involved in the processes of tolerance and immunosurveilance, Tregs are characterized by the nominal expression of the transcription factor FOXP3, and are induced by the presence of polarizing cytokines such as TGFB, IL-10 e IL-35. Is has been demonstrated high presence of Treg in peripheral blood of individuals with leukemia, although the mechanisms that produce this increase were not fully elucidated. Nonetheless, the promoter region of *FOXP3* gene has sensitive sequences to transcription factors activated by TGFB signaling pathway, and may have genetic polymorphisms, which have been linked to autoimmune diseases and some cancers. In this context, the role of the chemokine receptor CCR5 has been reviewed in the literature in order to assess the implications of this receptor in immunity against tumor microenvironment, their relationship with regulatory T cells, and the involvement of polymorphism rs333 (CCR5 delta 32) in susceptibility to ALL and its prognostic value for the development of disease risk, in an association study. The CCR5 may exert indirect effect on cancer progression, controlling the antitumor reponses, since its expression seems to promote tumor growth and contribute to metastasis. Described effects of a CCR5 antagonist have been able to inhibit tumor growth, suggesting its possible use as a therapeutic target for cancer. Furthermore, in a case-control association study, rs333 polymorphism of *CCR5* showed a similar distribution of heterozygous genotypes between control and leukemic groups, indicating no implications of this polymorphism in susceptibility to ALL and in relation to the risk status of disease. Additionally, it had been evaluated rs3761548 and rs2232365 polymorphisms in the promoter region of the *FOXP3* gene between patients with ALL and controls and the possible involvement in risk ALL, including association with the clinical outcome of patients. The allelic and genotypic frequencies were evaluated and there were no significant differences between the control and leukemic groups for rs3761548 and rs2232365 polymorphisms of the *FOXP3* gene. The presence of variant alleles and their genotypes was not associated with clinical progression and therefore these polymorphisms were not considered genetic susceptibility factors to ALL. Notwithstanding, we investigated the growth factor TGFB1 protein expression in the plasma of patients with ALL and cancer free controls, and aimed to associate genotypes of *FOXP3* polymorphisms with plasma TGFB1 levels.

Leukemic patients showed significantly reduced levels of TGFB1 (p=0.0007) compared to cancer free controls. This reduction was also observed in the comparison between the control and leukemic groups according to the genotypes of rs3761548 and rs2232365 polymorphisms of the *FOXP3* gene. Individuals with the homozygous variant genotypes for both polymorphisms showed higher levels of plasma TGFB1, although a significant difference was observed only for the polymorphism rs3761548 in the control group. These results support the hypothesis of deregulation in pathway activated by the TGFB in ALL, and this could operate modulatory effects on the expression of FOXP3 in the leukemic cells. Finally, this study suggests that genetic polymorphisms in the promoter region of *FOXP3* and TGFB1 plasma levels may be associated with the pathogenesis of ALL.

Key-words: CCR5. ALL. TGFB. FOXP3. Genetic polymorphisms.

OLIVEIRA, C.E.C. **POLIMORFISMOS GENÉTICOS DE *FOXP3* Y *CCR5*, Y NÍVELES PLASMÁTICOS DE TGFB1: IMPLICACIÓN EN LA PATOGÉNESIS DE LA LEUCEMIA LINFOIDE AGUDA INFANTIL Y JUVENIL**. 2013. 111 f. Tesis (Doctorado en Patología Experimental) - Universidad Estatal de Londrina. 2013.

RESUMEN

La leucemia linfoblástica aguda (LLA) es la neoplasia maligna más frecuente en los niños , que se origina a partir de un precursor de la linaje hematopoyético afectada de células B o T que ha sido objeto de una alteración genética que afecta el proceso normal de maduración, con cerradura la estimulación de la proliferación y diferenciación de la célula transformada. La acumulación de mutaciones en los genes, tales como los del sistema inmune y la regulación del ciclo celular, el desequilibrio entre la proliferación y la diferenciación de las células hematopoyéticas y los efectos de los factores de crecimiento sobre la leucemia pueden contribuir al desarrollo y el curso clínico de LLA. Las quimiocinas y sus receptores desempeñan papeles cruciales en la respuesta inmune y han sido implicados en el desarrollo de diferentes enfermedades y procesos patológicos, incluyendo el cáncer, en particular por la regulación del proceso de metástasis. El receptor de quimiocina CCR5 parece estar implicado en la patogénesis del cáncer, así como un polimorfismo deletéreo en el gen, nombrado CCR5 delta32 se asoció con un riesgo de desarrollar varias enfermedades. Por otra parte, el papel de las células T reguladoras (Treg) en el cáncer ha sido ampliamente investigado. Involucrados en los procesos de la tolerancia y la inmunovigilancia, células T reguladoras se caracterizan por la expresión nominal del factor de transcripción Foxp3, y se induce por la presencia de citocinas polarizantes tales como TGFB, IL-10 y IL-35. Se ha demostrado aumentada presencia de Treg en la sangre periférica em los pacientes leucemicos, aunque los mecanismos que causan este aumento no se ha aclarado completamente. Sin embargo, la región del promotor del gen *FOXP3* tiene secuencia sensible a los factores de transcripción activados por la vía de señalización del TGFB, y puede tener polimorfismos genéticos, los cuales han sido relacionados con enfermedades autoinmunes y algunos tipos de cáncer. En este contexto, el papel de los receptores de quimiocinas CCR5 se ha revisado en la literatura con el fin de evaluar las implicaciones de este receptor en la inmunidad contra el microambiente del tumor, su relación con las células T reguladoras, y la participación de lo polimorfismo rs333 (CCR5 delta32) en la susceptibilidad a LLA y su valor pronóstico para el desarrollo del riesgo de enfermedad en un estudio de asociación. El CCR5 para ejercer efecto indirecto sobre la progresión del cáncer, por el control de las respuestas antitumorales, ya que su expresión parece promover el crecimiento del tumor y contribuir a la metástasis. Efectos descritos de un antagonista de CCR5 fueron capaces de inhibir el crecimiento del tumor, lo que sugiere su posible uso como un objetivo terapéutico en cáncer. Además, en el estudio de asociación de casos y controles, el polimorfismo rs333 de *CCR5* mostró una distribución similar de los genotipos heterocigóticos entre el grupo de control y leucemia, que no muestran implicaciones de este polimorfismo en la susceptibilidad a LLA, y en relación con el estado de riesgo de la enfermedad. Por otro lado, se evaluaron los polimorfismos rs3761548 y rs2232365 en la región promotora del gen *FOXP3* entre los pacientes con ALL y controles y la posible implicación en riesgo en ALL, incluyendo en asociación con el resultado clínico de los pacientes. Se evaluaron las frecuencias alélicas y genotípicas y no hay diferencias significativas entre el grupo de control y leucemia para polimorfismos rs3761548 y rs2232365 del gen *FOXP3*. La presencia de alelos variantes y su genotipo no se asoció con la progresión clínica y por lo tanto estos polimorfismos no se consideraron factores de susceptibilidad

genética a LLA. Sin embargo, se determinó la expresión de la proteína del factor de crecimiento TGFB1 en el plasma de los pacientes con LLA y controles libres de cáncer, y hemos tratado de asociar los niveles plasmáticos con los genotipos de los polimorfismos del gene FOXP3. Pacientes leucémicos mostraron niveles significativamente reducidos de TGFB1 (p = 0,0007) en comparación a los controles sin cáncer. También esta reducción se observó en la comparación entre el control y leucemia de acuerdo con los genotipos de los polimorfismos rs3761548 y rs2232365 del gen *FOXP3*. Los individuos con los genotipos variantes homocigotos para ambos polimorfismos mostraron los niveles más altos de TGFB1 plasmático, aunque se observó una diferencia significativa sólo para el polimorfismo rs3761548 en el grupo de control. Estos resultados apoyan la hipótesis de que la desregulación en la vía activada por el TGFB en la LLA, y esto podría ejercer efectos moduladores sobre la expresión de FOXP3 en las células leucémicas. Por último, este estudio sugiere que los polimorfismos genéticos en la región promotora de *FOXP3* y los niveles plasmáticos de TGFB1 deben estar asociados con la patogénesis de la LLA.

Palabras clave: CCR5. LLA. TGFB. FOXP3 y Polimorfismos genéticos.

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LISTA DE ABREVIATURAS E SIGLAS

ABL	gene da leucemia murina de Abelson
AIDS	síndrome da imunodeficiência humana adquirida
AML	fator de transcrição relacionado ao Runt (conhecido como RUNX1)
ARF	proteína codificada por uma janela de leitura alternativa do gene CDKN2A (*alternate reading frame*)
BCL2	proto-oncogene 2 do linfoma de células B (*B cell lymphoma* 2)
BCR	região do cluster com sítio de quebra
CALLA	antígeno comum da leucemia linfoide aguda
CCL3	ligante 3 da família de quimiocinas CC
CCL4	ligante 4 da família de quimiocinas CC
CCL5	ligante 5 da família de quimiocinas CC
CCR5	receptor 5 de quimiocinas da família CC
CCR9	receptor 9 para quimiocinas da família CC
CD	marcadores de diferenciação (*cluster of differentiation*)
CDKN2A	gene do inibidor 2A de quinases dependente de ciclinas
CDKN2B	gene do inibidor B da quinase 4 dependente de ciclinas
CNFTR	receptor para o fator neurotrópico ciliar
CpG	citosina – fosfato – guanina
CXCL12	ligante 12 da família de quimiocinas CXC
CXCR4	receptor 4 para quimiocinas da família CXC
CXCR7	receptor 7 para quimiocinas da família CXC
EGIL	Grupo Europeu de classificação imunológica das Leucemias
FAB	Grupo Franco-Américo-Britânico
FCGR2	receptor II gama para porção Fc das imunoglobulinas G
FGFR1	receptor 1 para o fator de crescimento de fibroblastos
FOXP3	fator de transcrição Forkhead box P3
GATA3	fator de transcrição trans-ativador específico de células T
GNG12	proteína 12 ligadora do nucleotídeo guanina, subunidade gama (*Guanine Nucleotide-Binding Protein G(I)/G(S)/G(O) Subunit Gamma-12*)
HCM	hemoglobina celular média ou hemoglobina corpuscular média
HIV	vírus da imunodeficiência humana

HLA	antígeno leucocitário humano
HLF	fator leucêmico hepático
IFN γ	interferon gama
IgM	imunoglobulina M
IL-10	interleucina 10
IL-2	interleucina 2
IL-35	interleucina 35
INCA	Instituto Nacional do Câncer
INK4A e INK4B	proteínas regulatórias do ciclo celular que interagem com o CDK4 e CDK6.
ITCH:	ligase homóloga a proteína *itchy* E3 ubiquitina
LLA:	Leucemia Linfoide Aguda
LMA	Leucemia Mieloide Aguda
MIP-1α	proteína 1α inflamatória de macrófagos
MIP-1β	proteína 1β inflamatória de macrófagos
miR	micro RNA
MLL	gene da leucemia de linhagem mista
Myb	ativador transcricional Myb (de *avian myeloblastosis*)
MYC	proto-oncogene Myc (de *avian myelocytomatosis*)
NA	não aplicável
NFAT	fator nuclear de células T ativadas
NNAT	neuronatina
Notch	proteína homóloga a Notch loco neurogênico
OMS	Organização Mundial de Saúde
PAX5	fator de transcrição 5 da família paired box (PAX)
PBX1	homeobox1 da leucemia de células pré-B
PDGFRA	receptor A para o fator de crescimento derivado de plaquetas
PDGRFB	receptor B para o fator de crescimento derivado de plaquetas
RANTES	proteína regulada por ativação, secretada e excretada por células T
RNA	ácido ribonucleico
rs	referência de um polimorfismo genético atribuído pelo banco de dados dbSNP
SMAD3	proteína 3 homóloga dos produtos da família dos genes Mad de *Drosophila* e Sma de *C. elegans*

STAT4	proteína transdutora de sinal e ativadora da transcrição 4
STAT6	proteína transdutora de sinal e ativadora da transcrição 6
t	translocação cromossômica
TCF	fator de transcrição 3 (conhecido como E2A)
TdT	transferase deoxinucleotidil terminal
Teffs	células T efetoras
TEL	oncogene 6 variante ETS (conhecido como ETV6)
TGFB	fator de crescimento transformante beta
TIEG1	gene 1 precocemente induzido pelo TGFB
Tregs	células T regulatórias

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1 INTRODUÇÃO

As leucemias são um grupo heterogêneo de doenças hematológicas que diferem significativamente em termos das características morfológicas, imunofenotípicas, citogenéticas e moleculares. Estas características específicas refletem as diferenças no espectro de alterações biológicas envolvidas na transformação maligna, e/ou na variação dos níveis da hierarquia hematopoiética onde estes eventos ocorrem (GUENOVA, M. e BALATZENKO, G., 2013).

Como o próprio nome sugere (do grego “leukos” e “heima”, que significa sangue branco), as leucemias são rapidamente reconhecidas pelos efeitos na medula óssea normal e/ou no sangue periférico, comumente apresentando manifestações de hematopoese inefetiva e/ou consequências do acúmulo de células malignas (BROWN, C. M. et al., 2012).

Caracterizadas como neoplasias que acometem a medula óssea, apresentam proliferação medular anárquica e progressiva de células progenitoras hematopoiéticas, originárias das linhagens mieloide (eritrócitos, granulócitos e plaquetas) ou linfoide (FERNANDES, J.L. e MACIEL, F., 2011). No mielograma, revela-se profusa infiltração de células leucêmicas (ABDUL-HAMID, G., 2011).

A leucemia é a forma mais frequente de câncer em crianças menores de 14 anos (DIAMANTARAS, A. A. et al., 2012), somando cerca de 31% das neoplasias de ocorrência até os 15 anos, e atinge um máximo de 80 a 90 casos por milhão de pessoas entre os 2 e 3 anos, decaindo a 20 casos por milhão na faixa etária dos 8 aos 11 anos de idade, aumentado novamente após os 40 anos (MALONEY, K. W. et al., 2012). No Brasil, as estimativas da incidência do câncer, especialmente as leucemias, preveem de 4 a 5 novos casos em cada cem mil indivíduos para o ano de 2013 (INCA, 2011). As leucemias afetam indivíduos de ambos os sexos, sendo mais comuns em idosos (XIE, Y. et al., 2003).

Em geral, as neoplasias hematopoiéticas são classificadas de acordo com a linhagem, o grau de maturação e a integridade do desenvolvimento celular na medula óssea (hematopoese efetiva ou inefetiva) (SWERDLOW, S.H. et al., 2008). As cinco linhagens que originam as neoplasias mieloides incluem os granulócitos (neutrófilos, eosinófilos e basófilos), monócitos, eritrócitos, plaquetas (derivadas dos megacariócitos) e mastócitos. Por outro lado, as neoplasias que se originam da linhagem linfoide podem ser de células B ou de células T/NK (POPAT, U.R. e ABRAHAM, J., 2011). De acordo com a Organização Mundial de Saúde (OMS), a classificação das leucemias baseada nas linhagens acometidas pode ser determinada de acordo com o sumário apresentado no Quadro 1.

Quadro 1. Sumário da Classificação dos Tumores dos Tecidos Hematopoiéticos e Linfoides da OMS 2008

Neoplasias Mieloides
Neoplasias Mieloproliferativas Neoplasias Mieloides e Linfoides com eosinofilia e anormalidades de *PDGFRA*[1], *PDGRFB*[2] ou *FGFR1*[3] Neoplasias Mielodisplásicas/Mieloproliferativas Leucemia Mieloide Aguda e neoplasias de precursores relacionados Leucemias Agudas de linhagem ambígua
Neoplasias Linfoides
Neoplasias de Precursores Linfoides Neoplasias de Células B Maduras Neoplasias de Células T ou NK Maduras Linfoma de Hodgkin Desordens Linfoproliferativas associadas a Imunodeficiências Neoplasias de Células Dendríticas e Histiocíticas

[1]*PDGFRA*: receptor A para o fator de crescimento derivado de plaquetas; [2]*PDGRFB*: receptor B para o fator de crescimento derivado de plaquetas; [3]*FGFR1*: receptor 1 para o fator de crescimento de fibroblastos. Fonte: POPAT, U.R. e ABRAHAM, J., (2011).

A classificação é também determinada pelo curso clínico desencadeado pela doença. E desta forma, as leucemias podem ser classificadas como leucemias agudas e crônicas. As leucemias agudas são geralmente de rápida apresentação e progressão, frequentemente apresentando clones leucêmicos morfologicamente pouco diferenciados, enquanto as leucemias

crônicas possuem início insidioso, e geralmente são caracterizadas por células malignas com padrão de diferenciação normal (MILLER, R.G. et al., 2010).

O diagnóstico de um paciente com leucemia é realizado a partir da análise detalhada do número e da morfologia das células do sangue periférico. Inicialmente, a análise qualitativa, com a contagem absoluta e relativa dos eritrócitos, dos leucócitos e das plaquetas, revela informações importantes acerca das condições da hematopoese. Entretanto, a caracterização dos leucócitos em uma extensão (esfregaço) do sangue periférico e o contexto clínico do paciente são indispensáveis no diagnóstico inicial adequado (MCPHERSON, R.A. e PINCUS, M.R., 2011).

Avanços recentes nas técnicas de diagnóstico das leucemias promoveram um incremento significativo na sobrevida dos pacientes, principalmente em relação a leucemia linfoide aguda e aos linfomas linfoblásticos, pela intensificação das modalidades terapêuticas existentes, o transplante de medula óssea, e o escalonamento da dose das drogas quimioterápicas (SCHRAPPE, M. et al., 2002; REDDY, K. S. e PERKINS, S. L., 2004).

Desta forma, além do leucograma, a análise imunofenotípica por citometria de fluxo tornou-se uma ferramenta essencial no diagnóstico e na classificação das leucemias, permitindo a distinção da origem e dos estágios de diferenciação das células leucêmicas, provendo um ponto de referência para o tratamento clínico (QIU, Y. et al., 2009). Ainda, a análise do perfil de expressão gênica e de polimorfismos de base única tem permitido a identificação de novos subtipos leucêmicos, como a leucemia linfoide aguda de precursores de células T jovens (MULLIGHAN, C. G. et al., 2007; COUSTAN-SMITH, E. et al., 2009; MULLIGHAN, C. G. et al., 2009).

No contexto clínico, a contagem elevada de leucócitos e a idade ao diagnóstico são considerados indicadores confiáveis de prognóstico em pacientes leucêmicos tratados com quimioterapia convencional (PULLEN, J. et al., 1999; PUI, C. H. e EVANS, W. E., 2006).

Entretanto, marcadores imunofenotípicos tem levado a conclusões conflitantes em relação ao prognóstico (COUSTAN-SMITH, E. et al., 2009).

Assim, espera-se que as técnicas de diagnóstico mais sensíveis e específicas e a identificação de novos marcadores possam contribuir para o reconhecimento de casos de difícil tratamento e a caracterização da heterogeneidade biológica, o que pode prevenir a falha terapêutica nas leucemias.

1.1 LEUCEMIA LINFOIDE AGUDA

A leucemia linfoide aguda (LLA) é uma desordem maligna que se origina de um único precursor hematopoiético acometido para a linhagem de células B ou T. A aquisição de uma série de alterações genéticas interrompe os processos normais de maturação, levando ao bloqueio da diferenciação e à proliferação da célula transformada (GRAUX, C., 2011).

A incidência da LLA varia em todo o mundo; entretanto, existe maior frequência da doença em países com alto desenvolvimento sócio-econômico (MEJÍA-ARANGURÉ, J.M. et al., 2011), com exceção da alta frequência da LLA reportada em algumas cidades hispânicas, na Costa Rica e na Cidade do México (MEJIA-ARANGURE, J. M. et al., 2005; MEJIA-ARANGURE, J. M. et al., 2011).

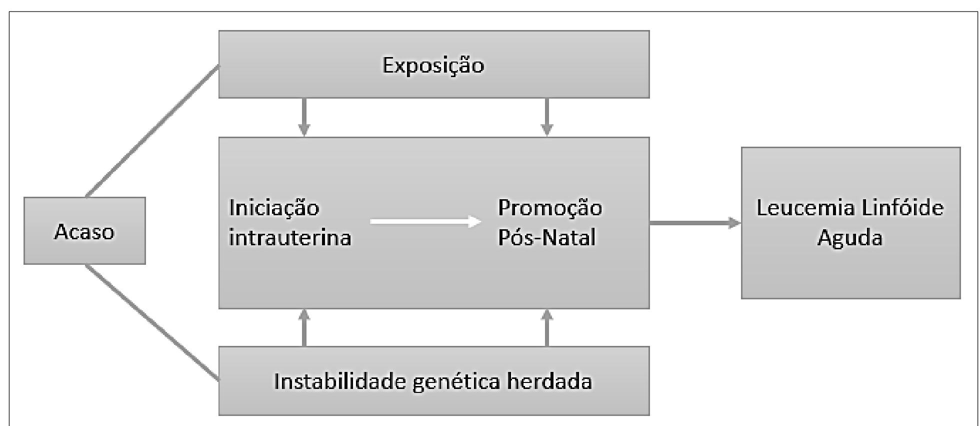
Nos Estados Unidos, cerca de 6000 novos casos de LLA foram estimados, na proporção de 1,3 homens para cada 1 mulher (SIEGEL, R. et al., 2012). Os pacientes são em sua maioria crianças, com 60% dos casos ocorrendo em indivíduos abaixo dos 20 anos de idade (PUI, C. H. et al., 2008; STANULLA, M. e SCHRAPPE, M., 2009; HUNGER, S. P. et al., 2012).

No Brasil, o Instituto Nacional do Câncer (INCA) estimou aproximadamente 4000 novos casos em mulheres (4 em cem mil) e quase 4600 casos em homens (5 em cem mil) para todas as leucemias, com incidência aumentada da LLA em crianças de 0 a 14 anos de idade (INCA, 2011).

Além disso, registros da incidência do câncer em 16 cidades de diferentes regiões brasileiras demonstraram uma estimativa média de 24,8 casos por milhão de habitantes e pico de incidência aos 3 anos de idade, para ambos os sexos (DE SOUZA REIS, R et al., 2011).

A LLA, como o câncer de forma geral, provavelmente origina-se das interações entre a exposição exógena e endógena, a susceptibilidade genética (herdada) e o acaso (Quadro 2) (INABA, H. et al., 2013). A patogênese da LLA ocorre a partir de lesões genéticas importantes em genes envolvidos com a diferenciação em linfócitos T ou B (PUI, C. H. et al., 2008).

Quadro 2. Causalidade para a Leucemia Linfoide Aguda infantil



Exposição exógena (p.ex. infecções) e endógena (p.ex. inflamação, estresse oxidativo), variação alélica em genes herdados e o acaso são fatores contribuintes para a história natural da LLA infantil. Fonte: INABA, H. et al., (2013).

O grande desafio é identificar elementos de exposição e variantes genéticas herdadas relevantes, e decifrar como e quando estes fatores contribuem para a história natural multi-etapas da LLA desde a iniciação (usualmente no útero) até a manifestação da doença (GREAVES, M. F. e WIEMELS, J., 2003).

1.1.1 Classificação da LLA

A LLA manifesta-se pela proliferação de blastos derivados da medula óssea que podem envolver o sangue periférico e órgãos sólidos, como os tecidos do sistema nervoso central. A porcentagem de blastos medulares requeridos para o diagnóstico das leucemias agudas foi tradicionalmente ajustado para 20% ou mais, e não requer uma porcentagem mínima de blastos quando as características morfológicas e citogenéticas estão presentes (ABDUL-HAMID, G., 2011).

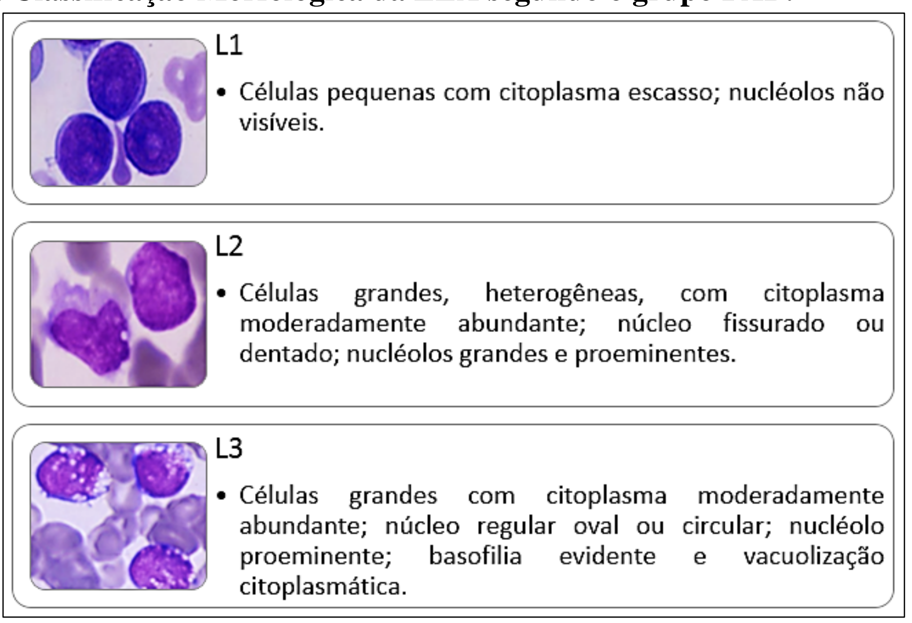
A definição de um consenso para a classificação da leucemia linfoide aguda ainda não foi determinada. Entretanto, três modelos de classificação são utilizados em diferentes países: (1) pela morfologia e citoquímica, suplementada pela imunofenotipagem, como proposto pelo grupo Franco-Américo-Britânico (FAB) (BENNETT, J. M. et al., 1976); (2) a classificação das leucemias agudas da OMS (HARRIS, N. L. et al., 1999); e (3) pela imunofenotipagem somente, como proposto pelo Grupo Europeu de Classificação Imunológica das Leucemias (EGIL) (BENE, M. C. et al., 1995).

De acordo com o grupo FAB, a LLA pode ser classificada como L1 (frequente em crianças), L2 (comum em adolescentes e adultos) e L3 (mais frequentes em pacientes com leucemia secundária ao linfoma de Burkitt). Estes três tipos são determinados de acordo com os critérios: a ocorrência de características citológicas individuais e o grau de heterogeneidade entre as células leucêmicas. As características consideradas são o tamanho celular, morfologia nuclear e da cromatina, quantidade de nucléolos, e o grau de basofilia e a presença de vacuolização no citoplasma (ABDUL-HAMID, G., 2011).

A OMS modificou a classificação da LLA de modo a considerar a elevada compreensão biológica e patogênese molecular da doença (Quadro 3). A nova classificação caracteriza estas

doenças heterogêneas baseando-se na imunofenotipagem e tipo celular acometido, considerando características moleculares (vide Quadro 1) (SWERDLOW, S.H. et al., 2008).

Quadro 3. Classificação Morfológica da LLA segundo o grupo FAB.



Fonte: ABDUL-HAMID, G., (2011).

O EGIL propõe a classificação das leucemias agudas com base na imunofenotipagem somente (Tabela 1). Esta classificação sugere um critério de padronização por definir a leucemia como mieloide, de linhagem T, de linhagem B ou bifenotípica, quando expressa antígenos mieloides em células linfoides, ou vice-versa (HOELZER, D. e GÖKBUGET, N., 2002).

A marcação imunofenotípica com anticorpo monoclonal deve atingir um limite mínimo de 20% para definir uma reação positiva para os blastos. Através destes marcadores foi possível observar que 75% das LLA em adultos acometem a linhagem celular B. Entre seus marcadores imunofenotípicos estão o CD19, CD20, CD22, CD24 e o CD79a (CAMPANA, D. e JANOSSY, G., 1988; HUH, Y. O. e IBRAHIM, S., 2000). Uma reação positiva para pelo menos dois dos três marcadores, sem outros marcadores adicionais detectáveis, identifica LLA pro-B. A presença do antígeno CD10 (CALLA) define o subgrupo comum da LLA. Casos com identificação adicional de IgM citoplasmático constituem o grupo pré-B, enquanto a presença na superfície de cadeias leves de imunoglobulina define a LLA-B madura (ABDUL-HAMID, G., 2011).

Tabela 1. Classificação das LLA pelo Grupo Europeu de Classificação das Leucemias (EGIL) com os respectivos marcadores imunofenotípicos e frequência relativa.

LLA de precursores de células B (HLA-DR, TdT, CD19 e/ou CD79a, e/ou CD22, e/ou CD34). Representa 75% dos casos adultos de LLA.		
a. LLA Pro B	HLA-DR, TdT, CD19	10%
b. LLA comum	CD10	50%
c. LLA Pré B	CD10 e Ig(cit)	10%
d. LLA-B madura	Ig(memb), TdT^- e $CD34^-$	4%
LLA de precursores de células T (TdT, CD3, CD34). Responsável por 25% dos casos de LLA adultos.		
a. LLA Pro T	$CD2^-$, $CD7^+$, $CD4^-$, $CD8^-$	7%
b. LLA Pré T	$CD2^+$, $CD7^+$, $CD4^-$, $CD8^-$	-
c. LLA T cortical ou tímica	$CD1a^+$, CD7, CD2, CD5, CD4 e CD8	17%
d. LLA T madura	CD3, CD2, CD7, CD4 ou CD8 TdT, CD34/$CD1^-$	1%

Fonte: ABDUL-HAMID, G., (2011).

Os marcadores de células T são o CD1a, CD2, CD3, CD4, CD5, CD7 e CD8. O CD2, CD5 e CD7 são os antígenos encontrados nas células imaturas, mas nenhum isoladamente representa uma linhagem específica, por isto o diagnóstico de LLA T deve demonstrar a molécula CD3 de superfície/citoplasmática (HOELZER, D. e GÖKBUGET, N., 2002).

As células leucêmicas da linhagem B ou T ainda podem adicionalmente expressar antígenos mieloides ou o antígeno de célula tronco CD34. Este tem baixa relevância ao diagnóstico, mas pode ser prognosticamente importante (DE WAELE, M. et al., 2001). Assim, foi proposto um sistema de escore pelo EGIL direcionando a caracterização das leucemias agudas de células B ou T, da leucemia mieloide aguda e das leucemias bifenotípicas (THALHAMMER-SCHERRER, R. et al., 2002).

A classificação ideal deve ser aquela capaz de reconhecer as características das células leucêmicas que apresentam diferenças biológicas únicas. Assim, é possível não apenas diferenciar mas também categorizar subtipos da LLA, permitindo um direcionamento da terapêutica.

1.1.2 Patofisiologia da LLA

A leucemia e os outros tipos de câncer compartilham uma mesma característica biológica: a clonalidade. As alterações moleculares requeridas para o desenvolvimento do câncer constituem um fenômeno raro, considerando-se o vasto número de células alvo susceptíveis a esta condição. Normalmente, uma única alteração genética raramente é suficiente para o desenvolvimento do tumor maligno (GALLEGOS-ARREOLA, M. P. et al., 2013).

A maioria das mutações em leucemias são adquiridas e ocorrem em um progenitor celular linfoide; menos frequentemente os genes mutados são herdados (1 a 5% das leucemias), e isto envolve uma anormalidade cromossômica numérica, como por exemplo, a trissomia do 21 (SEEWALD, L. et al., 2012). Desta forma, o desenvolvimento de uma doença hematológica maligna provavelmente envolve uma mutação em um gene crítico de proliferação, diferenciação e/ou sobrevivência celular em um progenitor hematopoiético (GREAVES, M., 2002).

Quando um oncogene é ativado por mutação, a proteína codificada está estruturalmente modificada e possui geralmente atividade transformadora aumentada, permanecendo em seu estado ativo e continuamente transmitindo seus sinais pela interação de tirosinas e/ou treonina quinases. Estes sinais induzem a proliferação celular continuada incessantemente (GALLEGOS-ARREOLA, M. P. et al., 2013).

Existem mutações que suprimem a função de genes e ocorrem nos genes supressores tumorais, como o *TP53*; entretanto, menos de 3% dos pacientes infantis e em média 9% dos pacientes adultos com LLA apresentam mutações no *TP53* (CHIARETTI, S. et al., 2013). Por outro lado, alguns autores tem encontrado alterações no número de cópias de genes em 50 regiões recorrentes na LLA, algumas pequenas com menos de 1Mb, entretanto ocorrem em sequências

codificadores de proteínas reguladoras do desenvolvimento linfoide em 40% dos casos de LLA de células B progenitoras (STOCK, W. et al., 2000; CHANG, J. S. et al., 2010).

As anormalidades cromossomais numéricas bem como rearranjos estruturais (translocações) ocorrem comumente na LLA (Tabela 2). Anormalidades citogenéticas importantes em precursores de células B estão associadas com LLA de pior prognóstico, incluindo t(9;22) ou cromossomo Filadélfia, com frequência diretamente proporcional a idade (RIX, U. et al., 2013); t(4;11), relacionada ao gene da leucemia de linhagem mista (*MLL*), comum na infância e também associada a leucemia mieloide (ARMSTRONG, S. A. et al., 2002; ARMSTRONG, S. A. et al., 2003); hipodiploidia (NACHMAN, J. B. et al., 2007); e trissomia do cromossomo 8 em adultos diagnosticados com LLA (BAKSHI, S. R. et al., 2012).

Tabela 2. Leucemia Linfoide Aguda e as Alterações Cromossômicas

Malignidade (subtipo FAB)	Anormalidade Cromossômica	Alterações Moleculares (genes envolvidos)	Frequência na LLA	Prognóstico
LLA	cariótipo normal	NA	20-45%	Favorável ou Intermediário
LLA	t(9;22)(q34;q11.2)	BCR-ABL1	5-20%	Desfavorável
LLA (L1 ou L2)	t(12;21)(p13;q22)	TEL-AML1	10-25%	Favorável
LLA (L1 ou L2)	t(1;19)(q23;p13.3)	TCF3-PBX1	2-5%	Favorável ou Intermediário
LLA	t(17;19)(q22;p13)	TCF3-HLF	1%	Desfavorável
LLA	t(8;14); t(2;8); t(8;22)	MYC-vários	1-2%	Desfavorável
LLA (L1 ou L2)	Hiperdiploidia (>50 cromossomos)	NA	10-25%	Favorável
LLA	Hipodiploidia (<45 cromossomos)	NA	1-5%	Desfavorável ou Intermediário

FAB: grupo Franco-Américo-Britânico; LLA: leucemia linfoide aguda; BCR: região do cluster com sítio de quebra; *HLF*: fator leucêmico hepático; L: subtipo de LLA; *MLL*: leucemia de linhagem mista; NA: não aplicável; *PBX1*: homeobox1 da leucemia de células pré-B; *TCF3*: fator de transcrição 3 (conhecido como E2A); *TEL*: variante 6 ets (conhecido como ETV6); *AML1*: conhecido como RUNX1; *ABL1*: gene da leucemia murina de Abelson. Fonte: adaptação de CHEN, J. et al., (2010).

Além disso, as alterações genéticas que se associam a LLA estão principalmente localizadas em sítios onde existem oncogenes, por exemplo: o oncogene *ABL* do cromossomo 9, acometido em certas translocações em LLA (CAZZANIGA, G. et al., 2011); o oncogene *MLL* do cromossomo 11, relacionado à LLA em crianças (AYTON, P. M. e CLEARY, M. L., 2001); a translocação t(8;14), associada à desregulação gênica do oncogene *C-MYC* (PALOMERO, T. et al., 2006); as mutações do gene supressor tumoral *TP53* (VILAS-ZORNOZA, A. et al., 2011); e ainda deleções e inversões, como por exemplo as deleções do fator de transcrição *PAX5*, presentes em pelo menos 30% das LLA de precursores de células B (HELTEMES-HARRIS, L. M. et al., 2011).

A metilação aberrante de ilhas CpG em regiões promotoras de genes foi identificada em linhagens de células de LLA, sendo considerada importante, uma vez que a metilação de dinucleotídeos CpG próximos aos sítios iniciadores da transcrição podem silenciar a expressão gênica (FEINBERG, A. P. e TYCKO, B., 2004). Desta forma, a hipermetilação de genes supressores tumorais e a hipometilação de oncogenes podem desencadear as leucemias.

No contexto da leucemogênese, a metilação aberrante de ilhas CpG na região promotora dos genes do inibidor de quinase 2B ciclina-dependente (*CDKN2B*), que codifica o supressor tumoral INK4B, e o *CDKN2A*, que codifica os supressores tumorais INK4A e ARF, são fenômenos bem descritos (MELKI, J. R. et al., 1999; GARCIA-MANERO, G. et al., 2009).

Outro mecanismo importante de desenvolvimento da LLA é a modificação da angiogênese (SCHNEIDER, P. et al., 2011) e da transdução de sinal na interação com receptores de tirosina quinases, e finalmente as moléculas reguladoras da apoptose (SANDA, T. et al., 2013), como é o caso do gene *BCL2*, o qual codifica uma proteína citoplasmática localizada na mitocôndria, e que aumenta a sobrevivência celular pela inibição da apoptose.

Ainda existem os casos de malignidades hematológicas secundárias, desenvolvidas a partir de complicações do tratamento do câncer. Usualmente, elas se manifestam como leucemias agudas ou síndromes mielodisplásicas e possuem frequência elevada, possivelmente pelo aumento do uso de agentes genotóxicos nas terapias antitumorais e pelo aumento da sobrevivência em outros tipos de câncer (SMITH, M. A. et al., 1996).

Evidências crescentes que suportam um processo multi-etapas na leucemogênese, com passos sequenciais e uma série de alterações em oncogenes, genes supressores tumorais e genes para microRNAs nas células tumorais (GREAVES, M., 2002; CROCE, C. M., 2008). Diferentemente dos genes envolvidos no desenvolvimento do câncer, os genes para microRNAs não codificam proteínas; seus produtos são pequenas moléculas de RNA (simples fita de 21 a 23 nucleotídeos) que reconhecem e ligam-se a sequências de nucleotídeos do RNA mensageiro (RNAm), complementares as sequências do microRNA, e então bloqueiam a tradução da proteína, regulando desta forma a expressão gênica (CALIN, G. A. et al., 2002; CROCE, C. M., 2008).

Vários microRNAs já foram implicados na patogênese da leucemia linfoide aguda (AKBARI MOQADAM, F. et al., 2013; BENETATOS, L. e VARTHOLOMATOS, G., 2013; DOU, L. et al., 2013; LI, X. et al., 2013b; LI, X. J. et al., 2013; YAN, J. et al., 2013). Recentemente, LI, X. et al., (2013a) identificaram um aumento na expressão dos microRNAs miR-708, miR210 e miR-181b em células LLA de precursores da linhagem B (LLA comum). E ainda, demonstraram que a expressão de miR708 está relacionada ao grupo LLA de alto risco, quando comparado aos grupos de baixo risco, por regular a expressão dos genes *CNFTR* (receptor para o fator neurotrópico ciliar), *NNAT* (neuronatina) e *GNG12* (proteína 12 ligadora do nucleotídeo guanina, subunidade gama).

Desta forma, pode-se concluir que a patofisiologia da LLA envolve mecanismos genéticos e ambientais complexos e em diferentes níveis, e que também deve existir uma relação de proximidade e complexidade entre estes fatores. Entretanto, o ponto chave na patofisiologia da

LLA é sua origem monoclonal, a proliferação celular descontrolada por estimulação autossustentada dos receptores para fatores de crescimento, a ausência de resposta aos sinais inibitórios, e a longevidade celular condicionada pela diminuição da apoptose (GALLEGOS-ARREOLA, M. P. et al., 2013). Assim, pode-se dizer que a LLA é o resultado de um processo de transformação maligna de células progenitoras da linhagem de linfócitos B ou T (PUI, C. H. et al., 2011).

As alterações moleculares requeridas para o desenvolvimento da leucemia são fenômenos raros, quando se considera o grande número de células alvo susceptíveis às modificações genéticas (GREAVES, M., 2002). É importante mencionar que quando se refere a origem do câncer, no caso da LLA, deve-se referenciar dois termos: a célula original e a célula tronco leucêmica. Atualmente, o conceito implica que células normais de origem distinta (célula original) adquirem a primeira mutação para promover o câncer, enquanto a célula tronco leucêmica irá disseminá-lo (VISVADER, J. E., 2011).

As leucemias apresentam marcada heterogeneidade em relação a morfologia celular, taxa de proliferação, lesões genéticas e, como resultado, resposta ao tratamento. Os mecanismos moleculares, compreendendo a heterogeneidade da neoplasia maligna, são aspectos importantes no estudo da biologia do câncer (VISVADER, J. E., 2011). E embora as alterações genéticas nas leucemias ocorram em grande parte por mutações somáticas, as alterações na linhagem germinativa podem contribuir para a predisposição ao câncer espontâneo ou familiar (hereditário) (CROCE, C. M., 2008).

1.1.3 Leucemia Linfoide Aguda e o Sistema Imunológico

Embora a etiologia da LLA seja pouco conhecida, o comportamento multifatorial da doença sugere que fatores de risco contribuam para o seu desenvolvimento, seja pela radiação ionizante, a quimioterapia e/ou as anormalidades cromossômicas (HAN, S. et al., 2010). Por outro lado, existem três hipóteses – (a) mistura populacional (STRACHAN, D. P., 1989), (b) infecção tardia (KINLEN, L. J., 1995) e (c) higiênico-sanitária (GREAVES, M., 2006) – que sugerem o envolvimento do sistema imunológico na etiologia da LLA.

As hipóteses da mistura populacional e infecção tardia sugerem que um sistema imune deficiente nos estágios iniciais do desenvolvimento humano podem causar anormalidades nas respostas imunológicas contra as infecções, permitindo que uma célula alterada se desenvolva. Ambas as hipóteses são similares a hipótese higiênico sanitária, que explica o surgimento da célula original pela frequência aumentada de alergias durante os primeiros anos de vida. Muitos estudos suportam as hipóteses das infecções e do sistema imunológico como fatores etiológicos da LLA (STRACHAN, D. P., 1989; KINLEN, L. J., 1995; GREAVES, M., 2006; CHANG, J. S. et al., 2010; HAN, S. et al., 2010; LINABERY, A. M. et al., 2010; CHANG, J. S. et al., 2012; WIEMELS, J., 2012), todavia, pouco é conhecido sobre o papel dos genes nesta etiologia.

A relação entre o sistema imune e a LLA é um processo complexo que envolve a interação de muitas células incluindo os leucócitos, as barreiras epiteliais, as proteínas do complemento, as citocinas, as células T auxiliares 1 (Th1), T auxiliares 2 (Th2), T regulatórias (Tregs) e T auxiliares 17 (Th17), além das moléculas CD28 (*cluster* de diferenciação 28, receptor para CD80 e CD86), FCGR2 (receptor II gama para porção Fc das imunoglobulinas G), GATA3 (fator de transcrição trans-ativador específico de células T), STAT4 (proteína transdutora de sinal e ativadora da transcrição 4), STAT6 (proteína transdutora de sinal e ativadora da transcrição 6) e muitas outras

(CHANG, J. S. et al., 2010). Variações nos genes destas células podem afetar o desenvolvimento e a função das respostas imunes, e então, aumentar a susceptibilidade à LLA (HAN, S. et al., 2010).

Neste contexto, poucos campos de estudo da imunologia sofreram evolução tão acentuada quanto o campo das quimiocinas. As quimiocinas foram estabelecidas como citocinas quimioatraentes em 1992 após o Encontro Internacional de Imunologia em Budapeste (LINDLEY, I. et al., 1993). Estas constituem uma grande família de mediadores inflamatórios e imunológicos, sendo proteínas secretórias produzidas por leucócitos e outras células, constitutivamente ou após indução, e exercendo seus efeitos localmente de forma autócrina ou parácrina (MOSER, B e WILLIMANN, K, 2004). Junto com seus receptores, desempenham funções chave na defesa imunológica pelo direcionamento e controle da migração, ativação, diferenciação e sobrevivência na fisiologia dos processos inflamatórios agudos e crônicos, bem como na desregulação patológica pela atração de células e estimulação de vários subgrupos específicos de leucócitos (VIOLA, A. e LUSTER, A. D., 2008).

Nas duas últimas décadas, as quimiocinas e seus receptores ganharam destaque científico, uma vez que desempenham funções cruciais no desenvolvimento de diferentes doenças e processos patológicos, incluindo a inflamação, as doenças autoimunes, as doenças infecciosas como o HIV/AIDS, e o câncer (MOSER, B e WILLIMANN, K, 2004; COMMINS, S. P. et al., 2010).

O sistema de quimiocinas demonstra significante redundância, uma vez que um receptor pode interagir com múltiplos ligantes, assim como um único ligante compartilha vários receptores de quimiocinas (ALLEN, S. J. et al., 2007). Entretanto, diferentes padrões de expressão para diferentes quimiocinas e receptores indicam que estas provavelmente possuem papéis distintos *in vivo* (MANTOVANI, A., 1999).

Aproximadamente 45 quimiocinas e 20 receptores de quimiocinas foram identificados e agrupados em quatro categorias (C, CC, CXC e CX_3C), classificadas pela localização dos resíduos de cisteína principais, próximo a região N terminal (KOIZUMI, K. et al., 2007).

O receptor 5 de quimiocinas CC (CCR5) pertence a superfamília de receptores triméricos transmembrana acoplados à proteína G ligadora do nucleotídeo guanina, que compreende a maior família de proteínas do corpo humano (RAPORT, C. J. et al., 1996). Ele exerce suas atividades via proteína G e interage com as quimiocinas RANTES (regulada por ativação, secretada e excretada por células T) ou CCL5 (quimiocina 5 da família CC), MIP-1α (proteína 1α inflamatória de macrófagos) ou CCL3 (quimiocina 3 da família CC) e MIP-1β (proteína 1β inflamatória de macrófagos) ou CCL4 (quimiocina 4 da família CC) (SAMSON, M. et al., 1996).

O CCR5 é expresso em monócitos, células dendríticas e na micróglia (SAMSON, M. et al., 1996) e em linfócitos Th1, que sintetizam interleucina-2 (IL-2), interferon-γ (IFNγ) e medeiam a ativação de fagócitos (SALLUSTO, F. et al., 1998). E além destes, recentemente foi observado que a subpopulação de células T, denominadas Tregs, e as Tregs de memória $CD103^+$ expressam o CCR5 (CHANG, L. Y. et al., 2012a).

Este receptor está envolvido na quimiotaxia de leucócitos aos sítios de inflamação (PROOST, P. et al., 1996), desempenhando importante papel no recrutamento de macrófagos, células T e monócitos (SPAGNOLO, P. et al., 2005). Ainda, é conhecido que o microambiente estromal deve exercer função ativa na patogênese do câncer, com a participação do CCR5 (MANES, S. et al., 2003; ZIMMERMANN, T. et al., 2010; GONZALEZ-MARTIN, A. et al., 2012b; GONZALEZ-MARTIN, A. et al., 2012a).

Neste contexto, foi demonstrado que o receptor CCR5 está expresso em células da biópsia de pele de pacientes com leucemia mieloide aguda (LMA). A produção autócrina de CCL3 e a expressão concomitante de CCR5 poderia facilitar a retenção de blastos da LMA na pele, em

conjunto com a interação CXCL12/CXCR4 (ligante 12 da família de quimiocinas CXC/receptor 4 da família de quimiocinas CXC). Ainda, as interações CXCL12/CXCR7 (receptor 7 da família de quimiocinas CXC) prolongaram a sobrevivência das células leucêmicas (FAAIJ, C. M. et al., 2010).

Um fator conhecidamente envolvido no desenvolvimento da linhagem linfoide de células hematopoiéticas é a via Notch (proteína homóloga a Notch loco neurogênico) (HOYNE, G. F., 2003; MAILLARD, I. et al., 2003; SANCHEZ-DOMINGUEZ, R. et al., 2012). Além disso, descobertas recentes demonstraram que mutações com ganho de função no receptor Notch1 são muito comuns na LLA e linfoma (ASTER, J. C. et al., 2008). MIRANDOLA, L. et al., (2012) investigaram o possível papel regulador da Notch1 na expressão e função dos receptores de quimiocinas CCR5, CCR9 e CXCR4, que apresentam funções na determinação das propriedades malignas e na localização de infiltrações extramedulares na leucemia. Estes autores sugeriram que anormalidades na via Notch1 desencadeiam aumento na expressão de CCR5 e CCR9 em blastos leucêmicos.

Uma mutação deletéria de 32 pares de bases (delta32) no gene *CCR5* causa o truncamento e a perda do CCR5 na superfície de células linfoides de indivíduos homozigotos. A deleção delta32 tipicamente resulta em retenção completa do CCR5 no retículo endoplasmático em homozigotos e expressão diminuída do receptor na superfície celular em heterozigotos (CHELLI, M. e ALIZON, M., 2001).

Este polimorfismo genético denominado rs333 (CCR5/delta32) tem sido associado ao aumento da susceptibilidade em diversas doenças como o lúpus eritematoso sistêmico (CARVALHO, C. et al., 2013), artrite reumatoide (LEE, Y. H. et al., 2013), esclerose múltipla (MOLLER, M. et al., 2013), diabetes (SONG, G. G. et al., 2013), hiperplasia prostática e câncer de próstata (ZAMBRA, F. M. et al., 2013). Entretanto, até o momento, o polimorfismo rs333 não foi investigado no contexto da LLA.

1.1.3.1 Leucemia Linfoide Aguda e as Células T regulatórias (Tregs)

A LLA é causada por anormalidades genéticas adquiridas por um precursor de células hematopoiéticas e acredita-se que a leucemia origine-se de um único precursor de linfócitos aberrante, que adquire a anormalidade inicial. Várias hipóteses tem sido propostas compreendendo os mecanismos causais da LLA infantil, embora seja estabelecido que a maioria, se não todos os tipos de câncer, seja resultante de uma combinação de exposição ambiental, influências modificadoras, susceptibilidade herdada e o acaso (GREAVES, M., 2006). Muito embora os aspectos biológicos e clínicos da LLA sejam bem documentados, pouco é conhecido sobre a susceptibilidade individual.

A heterogeneidade dos tipos celulares envolvidos na LLA é refletida na heterogeneidade de apresentações clínicas, prognóstico e possivelmente, de fatores de risco (ORSI, L. et al., 2012). Assim, a caracterização imunológica dos linfócitos revela dois grandes grupos de LLA: a LLA de células B e a LLA de células T (ONCIU, M., 2009).

Os linfócitos T apresentam importante função imunológica contra o crescimento tumoral e eles tornam-se Tregs na presença de estímulos apropriados e interações com as células tumorais. Estas células em conjunto com o fator de crescimento transformante beta (TGFB) são efetores imunológicos que agem de maneira coordenada, uma vez que o TGFB estimula o desenvolvimento das Tregs e também é uma das citocinas produzidas por elas e pelas células tumorais por si próprias, em um mecanismo com importantes implicações na progressão do tumor (ODA, J.M.M. et al., 2012).

As Tregs são uma linhagem de linfócitos T $CD4^{+}$ caracterizadas pela expressão do fator de transcrição Forkhead box P3 (FOXP3), e tem sido demonstrado que estas apresentam-se elevadas

no microambiente tumoral, de forma a limitar a inflamação e promover evasão da imunovigilância (BOS, P. D. e RUDENSKY, A. Y., 2012).

Em estudos prévios, uma alta porcentagem de Tregs foi encontrada nos tecidos tumorais e no sangue periférico de pacientes com vários tipos de câncer (MOON, H. W. et al., 2011), incluindo o carcinoma hepatocelular (ORMANDY, L. A. et al., 2005), câncer de mama (LIYANAGE, U. K. et al., 2002), carcinoma gástrico (KAWAIDA, H. et al., 2005), linfoma (YANG, Z. Z. et al., 2006), o câncer de pulmão e ovário (WOO, E. Y. et al., 2001; CURIEL, T. J. et al., 2004), e ainda, este aumento está comumente associado ao pior prognóstico e aos estágios mais avançados (ICHIHARA, F. et al., 2003; CURIEL, T. J. et al., 2004; SATO, E. et al., 2005). Recentemente, alguns estudos tem demonstrado alta porcentagem de Tregs em malignidades hematológicas, incluindo a LLA (KELLEY, T. W. e PARKER, C. J., 2010; MOON, H. W. et al., 2011; WU, C. P. et al., 2012).

O impacto funcional das Tregs no contexto dos tumores malignos em tecidos sólidos e naqueles baseados primariamente no sangue ou na medula óssea, como nas leucemias agudas, deve ser diferente. Entretanto, relativamente poucos estudos direcionaram tanto para o papel das Tregs em doenças leucêmicas quanto na utilidade da quantificação e avaliação das Tregs para predizer a evolução clínica (KELLEY, T. W. e PARKER, C. J., 2010).

Os avanços na biologia das Tregs culminaram com o estabelecimento do FOXP3 como um fator de transcrição crítico na caracterização e manutenção do fenótipo regulador destas células, tanto que deficiências neste marcador estão associadas ao desenvolvimento de doenças autoimunes (SAKAGUCHI, S. et al., 2010). Ainda, a perda da expressão e mutações somáticas no gene *FOXP3* humano foram encontrados em câncer de mama e próstata (ZUO, T. et al., 2007a; ZUO, T. et al., 2007b; WANG, L. et al., 2009), sugerindo que este deve agir como um supressor tumoral e que a inativação do *FOXP3* deve contribuir para o desenvolvimento do câncer (KIM, M. S. et al., 2011).

Polimorfismos tem sido descritos em várias regiões do gene *FOXP3*, como a região promotora, intrônica e exônica (ODA, J. M. et al., 2013). Uma região crítica para a iniciação e a regulação da transcrição do *FOXP3* é a sequência conservada promotora do *FOXP3*, localizada a montante do sítio de iniciação da transcrição (SAKAGUCHI, S. et al., 2008), onde dois polimorfismos, rs3761548 e rs2232365, foram descritos e associados ao risco e prognóstico em doenças multifatoriais como a psoríase (GAO, L. et al., 2010), rinite alérgica (ZHANG, Y. et al., 2012), vitiligo (JAHAN, P. et al., 2013), síndrome coronária aguda (YANG, Q. et al., 2013) e miastenia grave (ZHANG, J. et al., 2013).

Os polimorfismos no gene *FOXP3* poderiam modificar o FOXP3 funcionalmente ou quantitativamente (GAO, L. et al., 2010), levando a alterações na contagem das Tregs. Além disso, a identificação de polimorfismos de base única que afetem a função ou a expressão de genes e que contribuam para a susceptibilidade a LLA é importante, uma vez que poderiam ajudar na predição de risco individual e populacional, além de esclarecer mecanismos patofisiológicos relevantes à doença.

A expressão estável do *FOXP3* é claramente um pré-requisito para a manutenção das propriedades supressoras nas Tregs. A ação sinérgica dos sinais a jusante do receptor de células T (TCR), das moléculas coestimulatórias e dos receptores de citocinas é requerida para a transcrição ativa do FOXP3 (HUEHN, J. et al., 2009). Dentre as citocinas polarizadoras para a diferenciação das Tregs encontram-se o TGFB, a interleucina 10 (IL10), e a interleucina 35 (IL35); esta, recentemente identificada e mantenedora de potente função imunossupressora, principalmente na expansão das Tregs (OLSON, B. M. et al., 2013).

A diferenciação das Tregs a partir das células T $CD4^+$ é dirigida por sinais ativados em presença de TGFB (FU, S. et al., 2004). Além disso, a via de sinalização do TGFB tem papel definido na hematopoese normal, e está frequentemente alterada nas malignidades hematológicas,

incluindo a LLA (FORTUNEL, N. O. et al., 2000; LARSSON, J. e KARLSSON, S., 2005; RUSCETTI, F. W. et al., 2005; DONG, M. e BLOBE, G. C., 2006).

Os efeitos do TGFB sobre as células hematopoiéticas e a hematopoese são célula e contexto específicos. Os efeitos reguladores negativos da proliferação e positivos da diferenciação do TGFB foram estabelecidos *in vivo*, com a utilização de TGFB1 inibindo significativamente a proliferação de células progenitoras granulocíticas, eritrocíticas, megacariocíticas e de macrófagos (GOEY, H. et al., 1989), e retardando a recuperação hematológica após o tratamento de camundongos com a 5-fluorouracil, um antimetabólito e inibidor irreversível da timidilato sintase (JANSEN, R. et al., 1991).

MUROHASHI, I. et al., (1995) descreveram efeitos diferenciais do TGFB1 na proliferação de células normais e células hematopoiéticas leucêmicas. Os leucócitos de controles e pacientes com neoplasias hematológicas foram tratados com TGFB1 e diferentes fatores de crescimento. Eles demonstraram que o tratamento com TGFB1 inibiu a expressão dos oncogenes *C-MYC* (fator de transcrição Myc) e/ou *C-MYB* (ativador transcricional Myb) em blastos da leucemia mieloide aguda, promovendo a supressão do crescimento tumoral.

Ainda, foram descritas regiões sensíveis ao TGFB na sequência promotora do gene *FOXP3*. Neste contexto, VENUPRASAD, K. et al., (2008) mostraram que o gene 1 precocemente induzido pelo TGFB (*TIEG1*; também conhecido como KFL10) pode ligar-se ao promotor do *FOXP3* e cooperar com a ligase homóloga a proteína *itchy* E3 ubiquitina (ITCH) para induzir a expressão de FOXP3.

Além disso, o fator de transcrição induzido pelo TGFB SMAD3 (do inglês *mothers against decapentaplegic homologue 3*) foi associado ao controle da atividade de um acentuador (*enhancer*) intrônico do *FOXP3* em cooperação com o fator de transcrição NFAT (fator nuclear de células T

ativadas) (TONE, Y. et al., 2008). Isto indica que a sensibilidade a diferentes níveis de TGFB deve gerar variações na expressão de FOXP3.

Como o TGFB promove a expressão de FOXP3 permanece uma área de intensa investigação, com alguns fatores já descritos como responsáveis pela interação com o loco *FOXP3*, em resposta a sinalização pelo TGFB. Entretanto, uma via detalhada explicando esta interação ainda não foi evidenciada, principalmente em uma neoplasia hematológica, onde o TGFB conhecidamente regula a proliferação de células leucêmicas, e é talvez o mais potente regulador negativo endógeno da hematopoese (DOSEN-DAHL, G. et al., 2008).

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3 OBJETIVOS

OBJETIVOS GERAIS

- Analisar o envolvimento dos polimorfismos rs333 do gene CCR5, rs3761548 e rs2232365 do gene *FOXP3*, e a expressão proteica de TGFB1 na patogênese da Leucemia Linfoide Aguda Infanto-Juvenil.

OBJETIVOS ESPECÍFICOS

- Investigar a distribuição dos genótipos do polimorfismo rs333 (Delta32) do *CCR5* entre pacientes e controles de mesma faixa etária, livres de neoplasia, sua associação com a susceptibilidade ao desenvolvimento da Leucemia Linfoide Aguda Infanto-Juvenil e seu valor prognóstico em relação ao status de risco para a doença.

- Avaliar a distribuição alélica e genotípica dos polimorfismos rs3761548 e rs2232365 da região promotora do gene *FOXP3* entre pacientes e controles, o possível envolvimento na susceptibilidade ao desenvolvimento da Leucemia Linfoide Aguda Infanto-Juvenil, e a associação do risco-relativo entre os parâmetros clínicos de alto e baixo risco.

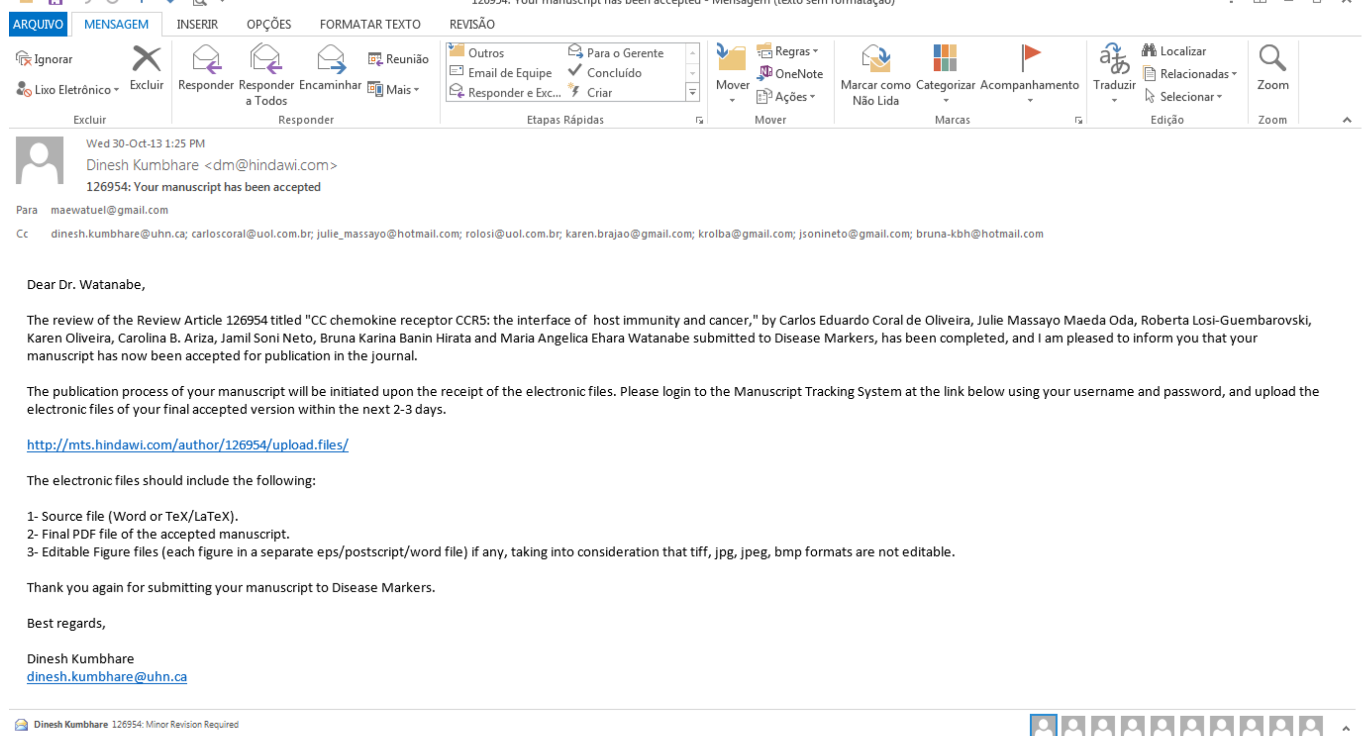
- Estimar a expressão proteica do TGFB1 no plasma de pacientes com Leucemia Linfoide Aguda e controles livres de neoplasia, verificar sua associação com os genótipos dos polimorfismos rs3761548 e rs2232365 do *FOXP3* e estabelecer uma correlação dos efeitos moduladores do TGFB1.

ARTIGO 1

TÍTULO: CC chemokine receptor CCR5: the interface of host immunity and cancer

PERIÓDICO: DISEASE MARKERS

ACEITE EM: 30/10/2013



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Dear Dr. Watanabe,

The review of the Review Article 126954 titled "CC chemokine receptor CCR5: the interface of host immunity and cancer," by Carlos Eduardo Coral de Oliveira, Julie Massayo Maeda Oda, Roberta Losi-Guembarovski, Karen Oliveira, Carolina B. Ariza, Jamil Soni Neto, Bruna Karina Banin Hirata and Maria Angelica Ehara Watanabe submitted to Disease Markers, has been completed, and I am pleased to inform you that your manuscript has now been accepted for publication in the journal.

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dinesh.kumbhare@uhn.ca

Dinesh Kumbhare 126954: Minor Revision Required

CC chemokine receptor CCR5: the interface of host immunity and cancer

Abstract

Solid tumors are embedded in a stromal microenvironment consisting of immune cells, such as macrophages and lymphocytes, as well as non-immune cells, such as endothelial cells and fibroblasts. Chemokines are a type of small secreted chemotactic cytokine and together with their receptors play key roles in the immune defense. Critically, they regulate cancer cellular migration and also contribute to their proliferation and survival. The CCR5 chemokine receptor is involved in leucocytes chemotaxis to sites of inflammation and plays an important role in the macrophages, T cells and monocytes recruitment. Additionally, CCR5 may have an indirect effect on cancer progression by controlling the antitumor immune response, since it has been demonstrated that its expression could promote tumor growth and contribute to tumor metastasis, in different types of malignant tumors. Furthermore, it was demonstrated that a CCR5 antagonist may inhibit tumor growth, consisting of a possible therapeutic target. In this context, the present review focuses on the establishment of CCR5 within the interface of host immunity, tumor microenvironment and its potential as a targeting to immunotherapy.

Keywords: CCR5, immunity, cancer, Tregs.

1. Introduction

Chemokines are a type of small secreted chemotactic cytokine. Together with their receptors they play key roles in the immune defense by directing and controlling the migration, activation, differentiation and survival in the physiology of acute and chronic inflammatory processes as well as in the pathological deregulations by attracting and simulating the various subsets of specific leukocytes (VIOLA, A. e LUSTER, A. D., 2008). Moreover, chemokines critically regulate cancer cellular migration and also contribute to their proliferation and survival (BALKWILL, F., 2004).

The identification of a large number of chemokine receptors and their selectivity characterization and expression have provided information on the traffic regulation of leukocytes in health and disease. They are expressed on different types of leukocytes constitutively or induced, depending on cell types (LOETSCHER, P. et al., 1996).

The chemokine system is often thought as showing significant redundancy since one receptor can bind multiple ligands, and conversely, a single ligand can bind several chemokine receptors (ALLEN, S. J. et al., 2007). However, differential spatio-temporal expression patterns for different chemokines and receptors in our body indicate that they probably have distinct roles *in vivo* (MANTOVANI, A., 1999). To date, about 45 chemokines and 20 chemokine receptors have been identified and are grouped into four categories (C, CC, CXC, and CX3C) based on the location of the main cysteine residues near the N-termini (KOIZUMI, K. et al., 2007). Chemokine receptors relay their signal through heterotrimeric G-proteins (O'HAYRE, M. et al., 2008).

The CC chemokine receptor 5 (CCR5) belongs to the trimeric guanine nucleotide-binding-protein-coupled seven-transmembrane receptor superfamily, which comprises the largest superfamily of proteins in the body (RAPORT, C. J. et al., 1996). Exerts its activity via

G protein and binds to the chemokines RANTES (CCL5), MIP-1 α (CCL3) and MIP-1 β (CCL4) (SAMSON, M. et al., 1996). This receptor is involved in the chemotaxis of leucocytes to inflammation sites (PROOST, P. et al., 1996) and plays important role in the recruitment of macrophages, T cells and monocytes in inflammation (SPAGNOLO, P. et al., 2005).

A common 32-base pair deletion mutation (delta32) in the *CCR5* gene causes truncation and loss of CCR5 on lymphoid cell surfaces of homozygotes. The delta 32 deletion typically results in complete retention of CCR5 in the endoplasmic reticulum within homozygous or diminished CCR5 expression in heterozygous on the cell membrane (CHELLI, M. e ALIZON, M., 2001). Also, it is interesting that the *CCR5* expression is under the control of a complexly organized promoter region upstream of the gene. The main transcriptional activity of the *CCR5* promoter region is contained within the downstream promoter P1, which is transactivated by the transcription factor cAMP responsive element binding protein 1 (CREB-1) (KUIPERS, H. F. et al., 2008). Study from WIERDA, R. J. et al., (2011) reveals that epigenetic mechanisms involving DNA methylation, histone acetylation and methylation modifications contribute to the transcriptional regulation of *CCR5* expression.

It is known that stromal microenvironment may play active roles in cancer pathogenesis with the participation of chemokine receptor CCR5. Although cancer tissue consists of various stromal cells, such as leukocytes, fibroblasts, and endothelial cells, there is little known about the driving forces of cells migration and infiltration into cancer tissue. In this context, the present review focuses on the establishment of CCR5 receptor within the interface of host immunity, tumor microenvironment, relation with T regulatory (Treg) cells in cancer and also its potential as a targeting to immunotherapy.

2. CCR5 receptor and the interface of host immunity

Chemokines presents the potential to stimulate T-cell activation, although the pattern of activation may differ for different chemokine–chemokine receptor interactions (NANKI, T. e LIPSKY, P. E., 2001).

Expression of chemokine receptors can define subtypes of T lymphocytes. Mature peripheral T lymphocytes express different chemokine receptors profiles depending on their functional phenotype. For example, T helper lymphocyte type 1 (Th1), which synthesize interleukin-2, interferon-γ and mediate phagocyte activation, express CXCR3 [chemokine (C-X-C motif) receptor 3], CCR2 (C-C chemokine receptor type 2) and CCR5. On the other hand, T helper lymphocytes type 2 (Th2) produce interleukin-4, interleukin-5, which mediate B lymphocyte antibody production, express CCR3, CCR4 and CCR2. These differences partly determine the type of immune response that will be deployed at an inflammation site (SALLUSTO, F. et al., 1998; WALLACE, G. R. et al., 2004). In this context, CCR5 regulates trafficking of lymphoid cells such as memory/effector Th1 lymphocytes, or myeloid lineage cells (e.g. monocytes, macrophages, immature dendritic cells) and microglia (PANZER, U. et al., 2005).

During recent years it has become evident that a subpopulation of T cells named T regulatory cells (Tregs) plays a major role in sustaining tolerance to self-antigens. Interesting expression of CCR5 was detected on Tregs (SCHLECKER, E. et al., 2012) and CD103+ effector/memory Tregs could express CCR5 (CHANG, L. Y. et al., 2012a). Newer fates for helper T cells continue to be identified, with differentiation based on production of their signature cytokines and master regulator transcription factors, such as Th9, Th17 and Th22 cells (NAKAYAMADA, S. et al., 2012; HIRAHARA, K. et al., 2013).

The importance of CCR5 for a proper immune response is very much dependent on the type of stimuli; moreover, in some cases, compensating mechanisms override the absence of CCR5 expression and function. It has been suggested that CCR5 has a far more important role in the immune response than in regulating the trafficking of immune cells (WEISS, E. M. et al., 2011).

CCR5 was found to possess various functions, other than chemotaxis. Activation of these receptors can induce a co-stimulatory effect and IL-2 secretion by T cells and IL-12 secretion by macrophages (ALIBERTI, J. et al., 2000; WONG, M. M. e FISH, E. N., 2003) and serves as anti-apoptotic signals for macrophages under viral infection (TYNER, J. W. et al., 2005).

The expression of this receptor is markedly upregulated upon T cell activation, which allows activated T cells to migrate towards sites of inflammation. However, although selective expression of CCR5 and CXCR3 on Th1 cells has been suggested (BONECCHI, R. et al., 1998), others demonstrated that CCR5 is equally present on all activated T cells independently of their functional Th polarization (SALLUSTO, F. et al., 1998).

Cytokines and chemokines have a crucial role in cancer-related inflammation with consequent, direct and indirect effects on the proliferative and invasive properties of tumor cells (AMEDEI, A. et al., 2012). Thus, in addition to the expression of the chemokine receptor CCR5, expression of ligands of this receptor is responsible for the attraction of lymphocytes to the tumor microenvironment.

CCR5 endogenous ligands include the main chemokines CCL3 (MIP-1α), CCL4 (MIP-1β), and CCL5 (RANTES). Their order of potency of metabolic activity is CCL3 > CCL4 = CCL5-(SAMSON, M. et al., 1996). Identification of suppressor factors produced by CD8+ cells that counter infection by certain HIV-1 strain infections (COCCHI, F. et al., 1995) previewed

the critical identification of CCR5 as being one of two chemokine receptor molecules that serve as co-receptors for HIV-1 entry (FENG, H. et al., 2012).

Macrophages, lymphocytes, and natural killer cells are the predominant cell types of the immune cells in cancer tissue. In addition, eosinophils, granulocytes, and B cells are present as minor immune cells in some cancers (VICARI, A. P. e CAUX, C., 2002). It is known that the chemokine CCL5 is highly expressed in cancer where it contributes to inflammation and malignant progression, but the question of which cancer cell-derived chemokines are friends and which are enemies remains unanswered.

3. CCR5 receptor and the interface of cancer

Chemokines are representative driving forces of leukocytes in the inflammatory process (YOSHIE, O. et al., 1997; YOSHIE, O., 2000), leading to the question whether or which chemokines secreted from cancer cells are specifically correlated with cancer progression via infiltration of leukocytes into cancer tissue. It has been postulated that cancer cell-derived chemokines increase the infiltrating immune cells in a particular cancer type, and promote or suppress cancer progression according to the type and immune effect potency of the infiltrating cells (KOIZUMI, K. et al., 2007).

Chemokines binding to their receptors can induce a serie of intra-cellular cascade reactions which may regulate the migration of cancer cells (BEN-BARUCH, A., 2006). Some reports indicate that the expression of chemokine receptors in many cancer cells is not random (ZLOTNIK, A., 2006) and may play a role in organ-specific metastasis. It has been also demonstrated that tumor cells can create autocrine gradients of chemokine receptors that guide their migration in a luring gradient under the influence of interstitial flow towards functional lymph nodes, even if lymphatic endothelial cells are absent; although the effect is greatly

amplified when both flow and cells are present. This process was denominated “autologous chemotaxis”, suggesting that chemokines are secreted by tumor cells themselves (SHIELDS, J. D. et al., 2007).

CCR5 may have an indirect effect on cancer progression by controlling the antitumor immune response (MANES, S. et al., 2003). Intermediate and strong CCR5 expression was significantly associated with non-metastatic colorectal cancer and correlated with both the infiltration of tumor margins with CD8+ T-lymphocytes and the absence of (lymphatic) metastasis. Weak or absent CCR5 expression was significantly associated with lymph node metastasis and advanced UICC (Union for International Cancer Control) stages III and IV. It is hypothesized that T-cell retention at the tumor site seems to be mediated by CCR5-dependent mechanisms of the immune and tumor cells. CCR5 might play a role during progression of colorectal carcinoma, possibly opposing to cancer progression. However, CCR5 expression of tumor cells might as well be an epiphenomenon regulated by specific chemokines (ZIMMERMANN, T. et al., 2010).

CCR5 expression on CD8+ T cells was necessary for their efficient activation and migration to the tumor site and for tumor killing; importantly, CCR5 must also be expressed by CD4+ T lymphocytes to achieve maximal CD8+ T cell effector function (GONZALEZ-MARTIN, A. et al., 2012b). Additionally, CCL5 was demonstrated to promote chemotaxis of monocytes, increasing MMP9 expression in MCF7 cells (AZENSHTEIN, E. et al., 2002) and angiogenesis, partly dependent on Vascular Endothelial Growth Factor (VEGF) secretion by endothelial cells (SUFFEE, N. et al., 2012), suggesting that the expression of CCL5 by cancer cells results not only in monocyte migration to the tumor site but also in protumorigenic activities of this chemokine and of proinflammatory cytokines that may facilitate metastasis formation and contribute to disease progression.

Metastasis represents the definite cause of 90% of deaths from solid tumors and it emerges from the somatic evolution of a genetically variegated cancer-cell population under the selective pressures of an environment that imposes tight rules on cellular behavior. The complete inefficiency of the metastatic process implies that healthy tissues naturally display a marked hostility toward invading tumor cells but certain cell lineages may express molecules that bias the metastatic efficiency to different target organs (GUPTA, G. P. e MASSAGUE, J., 2006).

It has been demonstrated that CCR5 expression could promote tumor growth and contribute to tumor metastasis. VAN DEVENTER, H. W. et al., (2005) showed that mice expressing CCR5 present enhanced local tumor growth and an impaired response to vaccine therapy compared to CCR5 knockout mice. The authors showed that CCR5 expression in stromal cells, but not hematopoietic cells, contributed to tumor metastasis. As an example, CCR5 is involved in metastatization of chondrosarcomas (TANG, C. H. et al., 2010) and in migration of oral cancer cells (CHUANG, J. Y. et al., 2009). Its expression correlates with the ability of aggressive natural killer cell leukemia cells to infiltrate into multiple organs (MAKISHIMA, H. et al., 2007), and with multiple myeloma cell growth, bone marrow homing and osteolysis (MENU, E. et al., 2006).

The literature describes the influence of CCR5 receptor, and also between its CCL5 chemokine, in different types of malignant tumors. Hodgkin Lymphoma Reed-Sternberg cells express CCR5, which upon engagement by different CCR5 ligands may directly contribute to clonogenic growth of these tumoral cells. Also, CCR5 was found to be expressed in many other Hodgkin lymphoma cell lines tested. Hodgkin Reed-Sternberg cells express a functional CCR5 capable of transducing proliferation signals and suggest that CCL5 and/or other chemokines released by Hodgkin Reed-Sternberg cells attract CD4+ T lymphocytes and eosinophils into

Hodgkin Lymphoma-involved lymphoid tissues and actively contribute to the formation of the typical Hodgkin Lymphoma cellular microenvironment (ALDINUCCI, D. et al., 2008).

The prognosis of patients with osteosarcoma distant metastasis is generally considered as very poor. WANG, S. W. et al., (2012) examined the migratory activity of human osteosarcoma cells through CCL5 gradient. They verified that CCL5-CCR5 interaction increases the expression of $\alpha v\beta 3$ integrin via MEK, ERK, p65, and NF-kB dependent pathway, contributing to migration of human osteosarcoma cells.

LIN, S. et al., (2012) have verified that CCR5 and CCL5 were highly expressed in breast cancer lymph nodes metastasis. Treatment with the cytokine TNF-□ increased the expression of CXCR2, CX3CR1, CCR9, and CCR5 in breast cancer MCF-7 cell line and caused a marked increase in the expression of CXCR2 and CCR5. This is also observed in the highly metastatic MDA-MB-231 cell line, although the levels of expression observed after cytokine stimulation are higher than those obtained in the MCF-7 cell line. Basal expression of a given chemokine receptor is not by itself a good marker of homing or aggressiveness and is subject to change by the microenvironment. There are cell sub-populations expressing different levels of chemokine receptors, which under a particular stimuli, change their expression levels and thus their aggressiveness. Recently, WANG, J. et al., (2013) demonstrated that CCR5 and CCL2 serum levels increased during the period from benign change to benign change with proliferation in samples of patients with breast mass, suggesting that they might be involved not only in the malignant process, but also benign process before atypia.

Human adipose-derived stem cells represent a cellular source of CCL5 which influences tumor cell migration and invasion of human breast cancer cell line MDA MB 231 in paracrine and autocrine fashion (PINILLA, S. et al., 2009). Bone marrow derived mesenchymal stem cells have been found to integrate into the tumor associated stromal and secrete CCL5 which

then acts in a paracrine way on the cancer cells to enhance their invasion (KARNOUB, A. E. et al., 2007). However, JAYASINGHE, M. M. et al., (2008) demonstrated that tumor-derived CCL5 expression alone does not make a significant contribution to disease progression, and points towards a role for host-derived CCL5 in breast cancer.

CCR5 was also expressed in skin biopsies of acute myeloid leukemia. The autocrine production of CCL3 together with CCR5 expression would facilitate the retention of the acute myeloid leukemia blasts in the skin. CCR5/CCL3 and CXCR4/CXCL12 interactions facilitate the retention of acute myeloid leukemia cells in the skin, and CXCR7/CXCL12 interactions subsequently prolong their survival (FAAIJ, C. M. et al., 2010).

Notch pathway is a well-known factor in the development of lymphoid lineage (HOYNE, G. F., 2003; MAILLARD, I. et al., 2003; SANCHEZ-DOMINGUEZ, R. et al., 2012). MIRANDOLA, L. et al., (2012) investigated the possible regulative role of Notch1 on the expression and function of chemokine receptors CCR5, CCR9 and CXCR4 that play a role in determining blast malignant properties and localization of extramedullary infiltrations in leukemia. In this context, these authors suggested that Notch1 pathway abnormalities trigger an increase of CCR5 and CCR9 expression on leukemic blasts.

In last years, it has become evident that a subpopulation of T cells, named T regulatory cells, represent an integral part of the immune system and play an important role in the interface of tumor and host immunity in breast cancer (WATANABE, Maria Angelica Ehara et al., 2010). Regulatory T cells (CD4+CD25+Foxp3+) are known to be involved in suppressing immune responses (BELKAID, Y. e ROUSE, B. T., 2005), and they also express CCR5 on their surface, enhancing migration to the peripheral inflamed tissues (YURCHENKO, E. et al., 2006; KANG, S. G. et al., 2007) and gravidic uterus (KALLIKOURDIS, M. et al., 2007) through interaction with CCR5 ligands.

4. CCR5 and Treg Cells: The Interface of Cancer

T cells present an important immunological response in tumor growth and they become Tregs in the presence of appropriate stimuli and interactions with tumor cells. These regulatory cells and the transforming growth factor beta (TGF-β) are immunological effectors that act in a coordinated manner, once that TGF-β stimulates the development of Tregs and also is one of the cytokines produced by them and by the tumor cells themselves, a mechanism that has important implications in tumor progression (ODA, J.M.M. et al., 2012).

Tumors evade immune destruction by actively inducing immune tolerance through the recruitment of CD4+CD25+Foxp3+ regulatory T cells. CD4+Foxp3+ Tregs, compared with CD4+Foxp3-effector T cells, preferentially express CCR5. Treg cells migration into the tumor microenvironment is mediated by the CCL5/CCR5 axis in pancreatic adenocarcinoma, and blockade of this pathway may represent a novel immunomodulatory strategy for the treatment of cancer. Disruption of CCR5 signaling in the tumor slows tumor growth through a Treg-mediated mechanism. Although, disruption of the CCL5/CCR5 axis skews migration of only about 20% of the CD4+ population (less Tregs, more Teffs). Its physiological importance is reflected in the effects on tumor growth (TAN, M. C. et al., 2009). Moreover, data from CHAKRABORTY, R. et al., (2012) which have explored the chemokine receptor expression profile and function, showed that *ex vivo* cultured Tregs retained the expression of CCR7 but dramatically downregulated CCR5 as compared with freshly isolated Tregs.

In addition, Chang, et al. investigated the anti-tumor ability of $CD103^+$ and $CD103^-$ Treg cells *in vivo* and *in vitro.* They found that the potent *in vivo* suppression ability of $CD103^+$ Tregs is due to the tissue-migration ability through CCR5 expression.

CD4+ Tregs infiltrate renal cell carcinoma, and their numbers predict poor prognosis (LIOTTA, F. et al., 2011). Tregs mediate effector T cell suppression and thereby could aid

tumor immune escape. CCR5, CXCR3, and CXCR6 are involved in the selective recruitment of T cells into renal cell carcinoma tissue. Thus, these chemokine receptors, along with CCR6, are involved in recruiting Tregs to the tumor site. Reducing Treg recruitment by blocking CCR5-, CXCR3-, and CXCR6-mediated homing risks preventing recruitment of naturally occurring antitumor T cells (OLDHAM, K. A. et al., 2012).

CHANG, L. Y. et al., (2012b) found that higher levels of CCL5 expression in human and murine colon tumor cells correlated with higher levels of CD8+ T cells apoptosis and infiltration of Tregs. In this study, TGF-β signaling blockade diminished apoptosis of CD8+ T cells, implicating TGF-β as an effector of CCL5 action. Their findings establish that CCL5/CCR5 signaling recruits Tregs to tumors and enhances their ability to kill antitumor CD8+ T cells, thereby defining a novel mechanism of immune escape in colorectal cancer.

TAN, M. C. et al., (2009) established that Treg migration to pancreatic adenocarcinoma is driven, at least in part, by CCR5 chemotaxis and further demonstrated that disruption of CCR5 chemotaxis might be a useful strategy for impairing recruitment of tumor-associated Tregs thereby slowing tumor growth. Considering this aspect, CCR5 could be a promising target for immunotherapy.

Elucidating the types of cells recruited and signal pathways involved in the crosstalk between tumor cells and stromal cells will help to identify novel strategies for cotargeting cancer cells and tumor stromal cells to suppress metastasis and improve patient outcome (KHAMIS, Z. I. et al., 2012).

Myeloid derived suppressor cells (MDSCs) are one of the key suppressor cells that regulate anti-tumor immune responses in conjunction with Tregs in tumor-bearing hosts (FUJIMURA, T. et al., 2010). Recently, it was demonstrated that mouse tumor-infiltrating granulocytic and monocytic (MO-MDSC) myeloid-derived suppressor cells expressed

increased levels of chemokines comprising the CCR5 ligands CCL3, CCL4, and CCL5, and they were responsible to recruit high numbers of Tregs. Intratumoral injection of CCL4 or CCL5 increased tumor-infiltrating Tregs, and deficiency of CCR5 led to their profound decrease, emphasizing the importance of CCR5 in the control of antitumor immune responses (SCHLECKER, E. et al., 2012).

A chemokine receptor antagonist of the CCL5 receptors, CCR5 and CCR1, was shown to inhibit experimental breast tumor growth, further implicating CCL5 as an important molecule in breast cancer (ROBINSON, S. C. et al., 2003). Regarding this aspect, Velasco-Velasquez, et al. (VELASCO-VELAZQUEZ, M. e PESTELL, R. G., 2013) used preclinical models and demonstrated that CCR5 promotes basal breast cancer subtype invasiveness and metastatic potential, while CCR5 inhibition abrogates them (VELASCO-VELAZQUEZ, M. et al., 2012).

Understanding cellular and molecular mechanisms of tumor pathogenesis is critically important for the development of new approaches to cancer treatment. Many studies showed that cellular structures and receptors play an essential role in molecular and physiological processes (DO VAL CARNEIRO, J. L. et al., 2009; AMARANTE, M. K. et al., 2011; AOKI, M. N. et al., 2011; DE OLIVEIRA, K. B. et al., 2012).

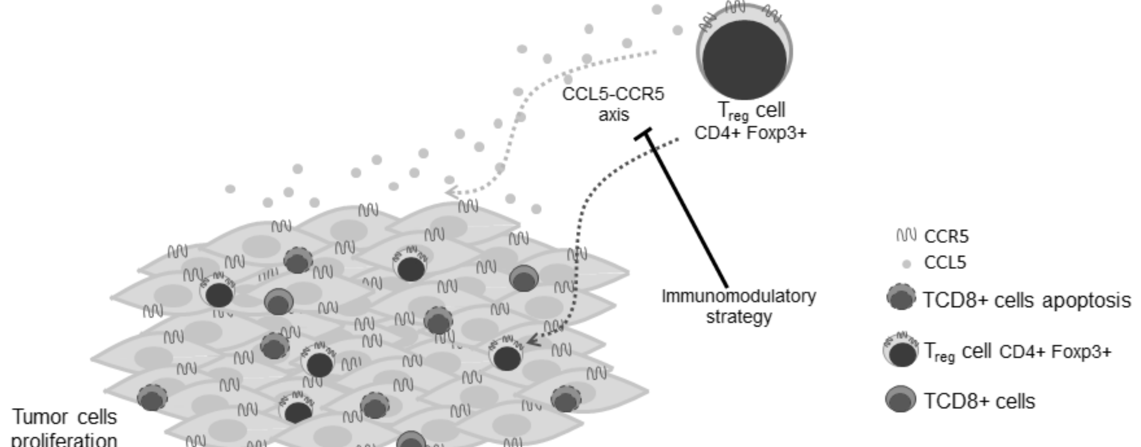

Figure 1. Schematic view of CCL5-CCR5 axis and Tregs involvement in tumor microenvironment. Tumors evade immune destruction by actively inducing immune tolerance through the recruitment of CD4+CD25+Foxp3+ regulatory T cells, which preferentially express CCR5 in the surface.

Cancer is a complex disease, where many alterations should be addressed in transformed cells. The CCR5 could influence on cancer formation, progression and prognosis, but this influence is not so sharp, in spite of recent results that have clarified many roles. Maybe in cancer development, Treg migration into the tumor microenvironment is mediated by the CCL5-CCR5 axis (Figure 1). In this context, enhancing their ability to regulate antitumor CD8+ T cells and other immune effector cells, encouraging cellular proliferation of tumor cells. Thus, blockade of this pathway may represent a novel immunomodulatory strategy for the treatment of cancer.

Action such as chemokine receptor and ligands may depend on interaction with signaling molecules by immunological or non-immunological cells, and this may be modified by genetic changes, mRNA and protein expression level. With a deeper understanding of the role of CCR5 in tumorigenesis process, it could be used as a molecular marker in diagnoses and prognoses, and even in treatment of cancer.

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ARTIGO 2

GENETIC POLYMORPHISM OF *CCR5* (rs333) DOES NOT INFLUENCE ACUTE LYMPHOBLASTIC LEUKEMIA SUSCEPTIBILITY OR RECURRENCE RISK

ABSTRACT

Acute lymphoblastic leukemia (ALL) is a malignant disorder that originates from one single hematopoietic precursor committed to B- or T-cell lineage. Ordinarily, these cells express CCR5 chemokine receptor, which directs the immune response to a cellular pattern and is involved in cancer pathobiology. Genetic polymorphism rs333 of CCR5 (delta32), results in a diminished expression of receptor, thus leading to impaired cell trafficking. The objective of the present study was to investigate the effect of *CCR5* chemokine receptor polymorphism rs333 in the pathogenesis of ALL in a childhood population of Southern Brazil. The distribution of the genotypes were studied in 79 patients and compared with 80 control subjects. Genotyping was performed using samples amplified by polymerase chain reaction with sequence specific primers (PCR-SSP). The Δ32/Δ32 deletion was not observed in any subject involved in the study. Heterozygous genotype was not associated to ALL risk (OR 0.7%; 95% CI 0.21-2.32; p>0.05), nor recurrence status of ALL (OR 0.86; 95% CI 0.13-5.48; p>0.05). The analysis of the genotypes demonstrated, for the first time, no significant differences in the frequency of the CCR5/Δ32 genotype between the ALL and control groups, indicating no effect of this genetic variant in the ALL susceptibility and recurrence risk.

KEYWORDS: CCR5, ALL, genetic polymorphism, recurrence risk.

INTRODUCTION

Leukemia is the most common type of childhood cancer, although overall incidence is rare. It accounts for 30% of all cancers diagnosed in children under 15 years. Within this population, ALL occurs approximately five times more frequently than acute myelogenous leukemia (AML) and accounts for approximately 78% of all childhood leukemia diagnoses (BELSON, M. et al., 2007). In Brazil, for 2012, INCA (National Cancer Institute) estimated approximately 8,510 new cases of leukemias. Among children and adolescents it is the most common type of cancer, accounting for 29% of all malignant tumors diagnosed and the highest incidence is in the age range of 1-4 years (INCA, 2011).

Chemokines and their receptors play a pivotal role in development of different types of cancer (BALKWILL, F., 2004). The chemokine system is often thought as showing significant redundancy since one receptor can bind multiple ligands, and conversely, a single ligand can bind several chemokine receptors (ALLEN, S. J. et al., 2007).

The CC chemokine receptor 5 (CCR5) belongs to the trimeric guanine nucleotide-binding-protein-coupled seven-transmembrane receptor superfamily, which comprises the largest superfamily of proteins in the body (RAPORT, C. J. et al., 1996). It exerts its activity via G protein and binds to the chemokines RANTES (CCL5), MIP-1α (CCL3) and MIP-1β (CCL4) (SAMSON, M. et al., 1996). This receptor is involved in the chemotaxis of leucocytes to inflammation sites (PROOST, P. et al., 1996) and plays important function in the recruitment of macrophages, T cells and monocytes in inflammation (SPAGNOLO, P. et al., 2005).

The importance of CCR5 for a proper immune response is very much dependent on the type of stimuli; moreover, in some cases, compensating mechanisms override the absence of CCR5 expression and function. It has been suggested that CCR5 has a far more important role in the immune response than in regulating the trafficking of immune cells (WEISS, E. M. et al.,

2011).

The specific biological and molecular mechanisms that account for the aggressiveness and poor therapy response of some acute lymphoblastic leukemia cases remains to be elucidated. Cytogenetic, molecular and genomic studies have shown that T cell acute lymphoblastic leukemia is a disease primarily caused by aberrant activation of the NOTCH1 signaling pathway (ASTER, J. C. et al., 2008). In this context, MIRANDOLA, L. et al., (2012) have investigated the possible regulative role of Notch1 on the expression and function of chemokine receptors CCR5, CCR9 and CXCR4 that are important in determining blast malignant properties and localization of extramedullary infiltrations in leukemia. Through interference RNA, they demonstrated that oncogenic Notch1 isoform in T cell ALL participates in controlling CCR5 and CCR9 expression and functions.

A common 32 base pair deletion (Δ32) in the *CCR5* gene causes truncation and loss of CCR5 on lymphoid cell surfaces of homozygotes. The Δ32 deletion typically results in complete retention of CCR5 in the endoplasmic reticulum within homozygous or diminished CCR5 expression in heterozygous on the cell membrane (CHELLI, M. e ALIZON, M., 2001).

It has been shown that CCR5 expression in stromal cells, but not hematopoietic cells, contributed to tumor metastasis. As an example, CCR5 is involved in metastatization of chondrosarcomas (TANG, C. H. et al., 2010) and in migration of oral cancer cells (CHUANG, J. Y. et al., 2009). Its expression correlates with the ability of aggressive natural killer leukemic cells to infiltrate into multiple organs (MAKISHIMA, H. et al., 2007) and with multiple myeloma cell growth, bone marrow homing and osteolysis (MENU, E. et al., 2006). *CCR5* studies have demonstrated the importance of the Δ32 mutation, particularly in the susceptibility to HIV infection (REICHE, E. M. et al., 2008), since CCR5 is a co-receptor in the primary stage of infection that is essential for the onset of the disease (GALVANI, A. P. e NOVEMBRE, J.,

2005).

Our research group has been studying polymorphic allelic variants related to the immune system and tumor development in different cancer types. Nevertheless, there are no data relating *CCR5* Δ32 polymorphism in ALL Brazilian population. Within this context, in the present report, we attempt to analyze genetic polymorphism of *CCR5* (rs333) in ALL patients from the Southern region of Brazil.

MATERIALS AND METHODS

Human subjects

Following approval from the Human Ethics Committee of the State University of Londrina, Paraná, Brazil (No. 214/09 – CAAE No. 0164.0.268.000-09), inclusion of the individuals to the study was conditioned by an obtained written informed consent form from parents regarding the use of their children and adolescents blood samples for research studies. Blood samples of seventy-nine ALL patients were enrolled in this study. Diagnostic criteria were based on the guidelines proposed by Hematology Department of the University Hospital. Recurrence risk status of ALL patients was evaluated through the GBTLI Protocol (Brazilian Group of Childhood Leukemia Treatment Protocol - 99) which is based on the Cancer Therapy Evaluation Program, proposed by the National Cancer Institute and take into account age at diagnosis, leukocyte count, immunophenotyping, involvement of tissues other than bone marrow and responsiveness to treatment. The control group comprised 80 healthy individuals free of neoplasia, well matched in relation to case group by age and gender.

Genomic DNA extraction

Genomic DNA was extracted from whole blood by Biopur Mini Spin Plus Kit (Biometrix Diagnostica, Curitiba, Brazil), according to manufacturer's instructions. DNA was eluted in 50µL of milliQ water and quantified by NanoDrop 2000c® Spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, USA) at a wavelength of 260/280 nm. Final preparation was stored at -20ºC and used as templates in polymerase chain reactions (PCR).

Optimization of PCR for CCR5

The method of genotyping (rs333) was optimized in the Laboratory of Study and Applications of DNA Polymorphisms of the State University of Londrina using specific primers for *CCR5* to amplify the genomic DNA. *Primer sense:* 5' ACC AGA TCT CAA AAA GAA 3' and *Primer anti-sense:* 5' CAT GAT GGT GAA GAT AAG CCT CA 3' GenBank sequence AF009962. Genotyping of CCR5-Δ32 was determined by PCR-SSP. The samples were amplified using 1.25 units Taq polymerase (Invitrogen™, Carlsbad, USA) in a Mastercycler Gradient Thermal Cycler (Eppendorf, Hamburg, Germany). CCR5 Δ32 PCR conditions were denaturation at 94˚C for 5 min, 35 cycles of 1 min at 94˚C, 1 min at 58˚C and 1 min at 72˚C, and 10 min of elongation at 72˚C. PCR products (225 bp or 193 bp) were analyzed on polyacrylamide gel (10%), stained with silver nitrate ($AgNO_3$).

Statistical analysis

Contingency tables and Fisher's exact test were used to calculate differences in genotype distributions and allele frequencies. $P<0.05$ was considered to indicate a statistically significant difference.

RESULTS

There were a total of 79 ALL patients and 80 healthy subjects (controls), all of whom were predominantly from Caucasoid population, due to European colonization. The mean age of controls and patients with ALL was 10.8 years± 5.65 and 8.7 years± 6.20 respectively. The patients and controls were well matched on gender, although there was a modest higher frequency of males in ALL group (53.16%) than in controls (48.75%). Fifty (63.29%) ALL patients were classified in high recurrence risk group and 29 (36.71%) in low recurrence risk group. The possible observed genotypes for *CCR5* rs333 polymorphism are shown in Figure 1.

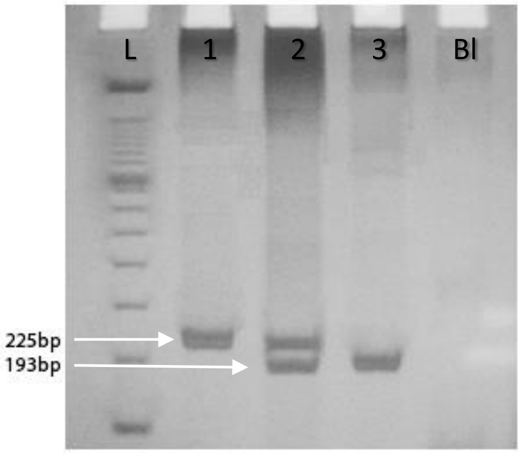

Figure 1. *CCR5* genotype profile. The PCR products were detected using silver staining method after polyacrylamide gel electrophoresis. *lane* L: DNA Ladder 100 bp; *lane* 1: CCR5 wild type homozygous genotype (CCR5/CCR5, 225 bp); *lane* 2: heterozygous genotype (CCR5/Δ32, 225 bp and 193 bp); and *lane* 3: variant allele homozygous genotype (Δ32/Δ32, 193 bp) and Bl represents Blank reaction.

Genotyping results showed any homozygous carriers for Δ32 deletion in both groups. The heterozygous CCR5/Δ32 genotype were observed in 8.75% (n=7) in controls and 7.5% (n=5) in ALL patients. To determine if there was a statistically significant increase risk of ALL development according to the *CCR5* genotypes, we conducted logistic regression analysis (Table 1), which showed that individuals with one copy of Δ32 variant allele did not exhibited association risk of ALL.

Table 1. **Genotype distribution and association study of *CCR5* rs333 polymorphism in control and ALL groups.**

	Genotypes		OR	95% CI	p
	CCR5/CCR5	CCR5/Δ32			
Control (80)	73 (91.25%)	7 (8.75%)	0.7	0.21-2.32	0.76
ALL (79)	74 (92.5%)	5 (7.5%)			
High risk (50)	47 (94%)	3 (6%)	0.86	0.13-5.48	1.00
Low risk (29)	27 (93.1%)	2 (6.9%)			

* Fisher´s exact test, p>0.05. OR: odds ratio; CI: confidence interval.

The *CCR5* genotypes distribution in ALL patients and controls were stratified by gender. Subgroup analyses revealed that the effect of gender were not significantly different among *CCR5* genotypes (female ALL vs. female control OR=0.52; 95%CI=0.09-3.07 and male ALL vs. male control OR=0.97; 95%CI=0.18-5.14).

In addition, we compared the *CCR5* genotype distribution in ALL patients classified in high risk or low risk, according to recurrence status. From five (7.5%) ALL Δ32 carriers, three were classified as high-risk patients. Although, association study between both recurrence status did not reach statistical significance.

DISCUSSION

Chemokines and their receptors are key regulators of immune activities and in parallel play conflicting roles in malignancy. While most combinations of these receptors and chemokines are active in cancer, many findings in the field have addressed the chemokine CCL5 and its cognate receptor CCR5 (WEITZENFELD, P. e BEN-BARUCH, A., 2013).

The gene variants of the chemokine and chemokine receptor genes associated with inflammation may be involved in cancer initiation and progression (KUCUKGERGIN, C. et

al., 2012). Considering the remarkable difference in CCR5/Δ32 allele frequency among worldwide populations, we aimed to survey the genetic variations in *CCR5* in 79 ALL patients and in 80 control individuals.

The age range of the patients in this study was as expected as for ALL occurrence, frequently in childhood and younger patients. Moreover, as previously mentioned, both sample groups were composed predominantly of Caucasian individuals from Southern Brazil. However, due to high degree of miscegenation of Brazilian population and the demand to use genetic markers for correct characterization of individuals (ARRUDA, V. R. et al., 1998; PARRA, F. C. et al., 2003) in our country, these data have not been explored in relation to the variants analyzed.

Chemokines and chemokine receptors are among factors that may influence ALL progression and localization (MIRANDOLA, L. et al., 2012). A 32-base pair nucleic acid deletion in *CCR5* exists and causes a frameshift mutation in the amino acids comprising the second extracellular loop. This deletion leads to premature truncation of the protein, disabling its ability to translocate to the membrane, impairs expression and ligand binding at the cell surface causing membrane receptor deficiency that may influence leukocyte trafficking (MAIER-MOORE, J. S. et al., 2013).

Based on this, we hypothesized that the ability of CCR5 to bind its ligands and signal recruitment of pathogenic T cells into target tissues may be impaired, thus imparting ALL protection. Although, there was no difference in the frequency of CCR5/CCR5 and CCR5/Δ32 genotypes between patient and control groups, showing that these alleles had no effect on the ALL susceptibility.

These results corroborated with the results of another studies which also identified no differences in the frequency of these alleles among healthy subjects and patients of southern

Brazilian population (BRAJAO DE OLIVEIRA, K. et al., 2007; MUXEL, S. M. et al., 2008; AOKI, M. N. et al., 2009) and worldwide (ZAFIROPOULOS, A. et al., 2004; DEGERLI, N. et al., 2005; GULERIA, K. et al., 2012).

Intensive multi-agent chemotherapy regimens and the introduction of risk-stratified therapy have substantially improved cure rates for children with ALL. Current risk allocation schemas are imperfect, as some children are classified as lower-risk and treated with less intensive therapy relapse, while others deemed higher-risk are probably over-treated (TEACHEY, D. T. e HUNGER, S. P., 2013). In this context, genetic polymorphisms in chemokine receptors could predict outcome and be considered an independent risk factor to stratify and allocate therapy in ALL.

Among patients with ALL (n=79), twenty nine (36,71%) were considered of low risk and fifty (63.29%) of high risk, according to the clinical and laboratorial findings at diagnosis, as defined by GBTLI LLA-99 protocol (INCA, 2011). When the genotype data were analyzed for stratified group of ALL, as low or high recurrence risk, the results indicated that the presence of Δ32 did not influence this clinical parameter. A recent study conducted by our research group have not found association between TP53 and CXCL12 genetic polymorphisms and ALL recurrence risk status (DE LOURDES PERIM, A. et al., 2013).

Nevertheless, the present study showed no difference in the frequency of CCR5/Δ32 genotype between childhood ALL patient and control groups, as well rs333 polymorphism does not influence recurrence risk of ALL. Further studies are required to elucidate the role of *CCR5* and its polymorphism in ALL pathogenesis.

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ARTIGO 3

FOXP3 POLYMORPHISMS rs3761548 AND rs2232365 IN BRAZILIAN PATIENTS WITH ACUTE LYMPHOBLASTIC LEUKEMIA

Abstract

Acute lymphoblastic leukemia (ALL) is the most common childhood malignancy and accounts for approximately 80% of leukemia in pediatric age group. Regardless of unknown etiology and pathogenesis of ALL, a lack of immune stimuli during childhood was related to leukemia susceptibility. T regulatory cells (Tregs), which express the transcription factor forkhead box P3 (FOXP3), play a major role in sustaining tolerance to self-antigens, and are involved with tumor cell evasion of immunosurveilance. High percentage of Tregs was described in several types of cancer, including ALL, and they were usually associated with a poor prognosis. The identification of *FOXP3* single nucleotide polymorphisms (SNPs) in ALL patients is important as it may help predicting individual and population risk and clarify pathophysiological mechanisms relevant to this disease. The aim of this study was to evaluate the association between two polymorphisms of *FOXP3* gene and ALL susceptibility among southern Brazilian population. We performed a case-control study involving 61 Brazilian children and adolescents with ALL and 73 cancer-free subjects. All subjects were genotyped for rs3761548 and rs2232365 *FOXP3* polymorphisms using allele specific-polymerase chain reaction (AS-PCR) assay. Genotype distribution and allele frequency were not associated with ALL in male nor in female subjects (p values ranged from 0.36 to 1.00). There was no association between alleles or genotypes of *FOXP3* polymorphisms and ALL development risk nor clinical outcome ($p>0.05$). No statistical differences were observed when the combination of simultaneous presence between variant alleles of FOXP3 polymorphisms were assessed. Our results demonstrate for the first time that polymorphisms in the promoter region of *FOXP3* gene are not a genetic susceptibility factor for ALL in south Brazilian population.

KEYWORDS: Acute lymphoblastic leukemia, FOXP3, genetic analysis, Tregs.

Introduction

Leukemia is the most frequent form of cancer in children less than 14 years (DIAMANTARAS, A. A. et al., 2012), accounting for 31% of cancers before 15 years, and reaches a maximum of 80 to 90 cases per million at 2 to 3 years of age, dropping to 20 cases per million at 8 to 11 years of age (MALONEY, K. W. et al., 2012). In Brazil, estimate rates of leukemia for 2012 and 2013 were ranged from 4 to 5 cases per hundred thousand people (INCA, 2011).

Acute lymphoblastic leukemia (ALL) is characterized by the monoclonal and/or oligoclonal proliferation of hematopoietic precursor cells of the lymphoid series within the bone marrow (BM). Nonetheless, it is the commonest childhood malignancy, accounting for approximately 80% of leukemia in the pediatric age group, but its etiology is unknown (STILLER, C. A. e PARKIN, D. M., 1996).

It is not well established which multiple risk factors contribute to leukemogenesis in childhood. Recently the deficit of normal immune stimulation in early childhood was considered a suspected risk factor for childhood ALL (GREAVES, M., 2006; LINABERY, A. M. et al., 2010; CHANG, J. S. et al., 2012; WIEMELS, J., 2012). Conflicting studies demonstrated that a lack of priming by infections during early childhood may cause a dysregulated immune response to infections later in childhood, leading to the development of childhood leukemia, particularly ALL, in susceptible individuals (Greaves' "delayed infection" hypothesis) (GREAVES, M., 2006). These results suggested that pathogenesis of childhood ALL may involve an altered component of immune system.

Regulatory T cells (Tregs), a CD4 T cell lineage characterized by the expression of forkhead box P3 (FOXP3) transcription factor, have been shown to be increased in tumor microenvironment in order to limit inflammation and evade immunosurveilance (BOS, P. D. e RUDENSKY, A. Y., 2012). In previous studies, a high percentage of Tregs was found in tumor

tissues and peripheral blood (PB) from patients with various cancers (MOON, H. W. et al., 2011), including hepatocellular carcinoma (ORMANDY, L. A. et al., 2005), breast (LIYANAGE, U. K. et al., 2002), gastric (KAWAIDA, H. et al., 2005), lymphoma (YANG, Z. Z. et al., 2006), lung and ovarian cancer (WOO, E. Y. et al., 2001; CURIEL, T. J. et al., 2004), and normally it was associated with poor prognosis and more advanced stage (ICHIHARA, F. et al., 2003; CURIEL, T. J. et al., 2004; SATO, E. et al., 2005). Recently, some studies have demonstrated high percentage of Tregs in hematologic malignancies, including ALL (KELLEY, T. W. e PARKER, C. J., 2010; MOON, H. W. et al., 2011; WU, C. P. et al., 2012).

Although various signals that induce the expression of *FOXP3* have been identified, the precise mechanisms by which the expression of this protein is controlled in Treg cells are not well understood. So far, it has been established that synergistic action of signals downstream of T-cell receptor (TCR), co-stimulatory molecules and cytokine receptors are required for the active transcription of *FOXP3* (HUEHN, J. et al., 2009).

In many cancers, genetic polymorphisms have been investigated and they may determine individual susceptibility to leukemia development (DE OLIVEIRA CAVASSIN, G. G. et al., 2004; DE OLIVEIRA, C. E. et al., 2007; DE LOURDES PERIM, A. et al., 2013). Polymorphisms of *FOXP3* gene could change FOXP3 functionally or quantitatively (GAO, L. et al., 2010), therefore leading to altered counting $CD4^{+}CD25^{+}$ Tregs.

The identification of single nucleotide polymorphisms (SNPs) that affect gene function or expression and contribute to ALL susceptibility is important as it may help to predict individual and population risk and clarify pathophysiological mechanisms relevant to this neoplasia. Thus, for the first time, we assessed two single-nucleotide polymorphisms of *FOXP3* gene (rs3761548 and rs2232365) in order to analyze their involvement in susceptibility of childhood ALL, as well as the correlation with low or high-risk clinical parameter.

Methods

Study Population

Following approval from the Human Ethics Committee of the State University of Londrina, Paraná, Brazil (N°. 214/09), inclusion of the individuals to the study was conditioned by an obtained written informed consent form from parents regarding the use of their children and adolescents blood samples for research. Blood samples were collected from 61 patients with ALL seen in the University Hospital of Londrina and Londrina Cancer Institute between March 2009 and November 2012. During the same time, 73 unrelated age-matched individuals, with no evidence or history of leukemia or any other cancer, were enrolled as the control group.

The ALL diagnostic criteria were based on the guidelines proposed by Hematology Department of the University Hospital. Clinical outcome was evaluated through the GBTLI Protocol (Brazilian Group of Childhood Leukemia Treatment Protocol - 99) which is based on the Cancer Therapy Evaluation Program, proposed by the National Cancer Institute (INCA, 2011). Through this protocol, patients were classified into low or high-risk group considering age at diagnosis, leukocyte count, immunophenotyping, involvement of tissues other than bone marrow and responsiveness to the treatment. Peripheral blood samples from patients and controls were frozen at -20°C until analysis. Most of our sample, both patients and controls, were predominantly Caucasian, a prevalent population in southern Brazil due to European colonization.

DNA extraction

Genomic DNA was isolated from 200µL peripheral blood cells using the Kit Mini Spin Plus (Biopur, Biometrix Diagnostica Ltda, Curitiba, Brazil) according to the manufacturer protocol. After precipitation with ethanol, the pellet was resuspended in 50µL of Biopur Kit specific buffer and quantified by NanoDrop 2000c (Thermo Fisher Scientific Inc., Massachusetts, U.S.A.) for later use in genotyping analyses.

Genotyping

Allele specific-polymerase chain reaction (AS-PCR) analysis was performed to determine the genotypes of two polymorphisms of *FOXP3*, as described previously (GAO, L. et al., 2010), with modifications. Amplification was performed in Mastercycler Gradient Thermalcycler (Eppendorf, Hamburg, Germany) with 100ng of genomic DNA, 150pmol of each primer, 100µM total dNTP, 1.5mM $MgCl_2$, 1X PCR Buffer and 1.25 units of Taq DNA polymerase (Invitrogen, Brazil). The PCR conditions were an initial preheating step for 10min at 94°C. Subsequent 35 cycles consisted of 94°C for 30sec, 67°C for 30sec, and 72°C for 1min, followed by 1 cycle of 72°C for 10min. The 334 bp (A) e 333 bp (C) fragments encompassing the C to A polymorphic site in the promoter region of *FOXP3* gene (rs3761548) were amplified using specific primers 5'-CTGGCTCTCTCCCCAACTGA-3' and 5'-ACAGAGCCCATCATCAGACTCTCTA-3' for A allele and 5'-TGGCTCTCTCCCCAACTGC-3' and 5'-ACAGAGCCCATCATCAGACTCTCTA -3' for C allele. The A to G polymorphism (rs2232365) was amplified using specific primers 5'-CCCAGCTCAAGAGACCCCA -3' and 5'-GGGCTAGTGAGGAGGCTATTGTAAC -3' for A allele and 5'-CCAGCTCAAGAGACCCCG -3' and 5'-GCTATTGTAACAGTCCTGGCAAGTG -3' for G allele, revealing a 442 bp and 427 bp PCR products, respectively. The amplicons were analyzed by 10% acrylamide gel electrophoresis visualized after silver nitrate staining.

Statistical Analysis

Odds ratio (OR) was performed to obtain association study for risk of ALL development and Relative Risk (RR) for clinical outcome, at 95% confidence intervals (CIs). OR, RR and 95% confidence intervals were assessed by logistic regression using Prism 5 for Windows (GraphPad Software, U.S.A.). For each candidate SNP, we followed the analytical approach reported for each locus, assuming allelic test and genotypic test. The combined genotypes data

were further stratified by subgroups of clinical outcome and two-sided tests of statistical significance at the 5% level (Fisher exact test) were performed using the Prism 5 for Windows (GraphPad Software, U.S.A.).

Results

The age of ALL patients and controls ranged between 0 and 21 years (means ALL: 9.65± 6.9 years; control: 11.25± 5.5 years). There were approximately equal percentage of males and females in both groups (control: 53.4% males and 46.6% females vs. ALL: 52.5% males and 47.5% females). Forty-two ALL patients (68.85%) were classified as high-risk group and 19 (31.15%) as low-risk group.

Since *FOXP3* is located on X chromosome, separate genotyping for female and male subjects was performed. For all female groups the genotype did not differ from the theoretical distribution given by the Hardy-Weinberg equilibrium (p>0.05). *FOXP3* SNPs did not show association with the disease within male group, as shown in Table 1.

Table 1. Hemizygous genotype frequencies of *FOXP3* SNPs in control and ALL (cases) male subjects.

FOXP3 SNPs	Genotype n(%)		OR (95% CI)	*p* value*
rs3761548	C	A		
Cases (n=32)	20 (62.5)	12 (37.5)	1.1 (0.41-2.80)	1.00
Controls (n=39)	25 (64.1)	14 (35.9)		
rs2232365	A	G		
Cases	17 (53.1)	15 (46.9)	1.3 (0.52-3.40)	0.63
Controls	18 (46.2)	21 (53.8)		

* Fisher´s exact test, p>0.05. OR: odds ratio; CI: confidence interval.

Genotype distribution and allele frequencies of female group are summarized in Table 2. Using AS-PCR analysis, we demonstrated CC genotype in 50.0% and 65.5%, heterozygosity in 35.3% and 27.6%, and homozygous genotype for the variant allele in 14.7% and 6.9% of healthy controls and ALL patients, respectively, for *FOXP3* gene polymorphism rs3761548. In relation to rs2232365, ALL patients demonstrated slightly increased common and variant

homozygous genotype distribution.

Table 2. Allelic and genotype frequencies in female subjects of the case-control study between *FOXP3* polymorphisms and ALL Risk[a]

		Cases	(%), n=29	Controls	(%), n=34	OR	CI (95%)
Allele Frequency							
rs3761548	C	46	(79.3%)	46	(67.6%)	1.00	(Reference)
	A	12	(20.7%)	22	(32.4%)	0.55	(0.24-1.20)
rs2232365	A	32	(55.2%)	37	(54.4%)	1.00	(Reference)
	G	27	(44.8%)	31	(45.6%)	1.00	(0.50-2.00)
Genotype							
rs3761548	CC	19	(65.5%)	17	(50.0%)	1.00	(Reference)
	CA	8	(27.6%)	12	(35.3%)	0.60	(0.20-1.80)
	AA	2	(6.9%)	5	(14.7%)	0.36	(0.06-2.10)
rs2232365	AA	10	(34.5%)	10	(29.4%)	1.00	(Reference)
	AG	12	(41.4%)	17	(50.0%)	0.71	(0.22-2.20)
	GG	7	(24.1%)	7	(20.6%)	1.00	(0.26-3.90)
Allele carriers							
rs3761548	CC	19	(65.5%)	17	(50.0%)	1.00	(Reference)
	CA + AA	10	(34.5%)	17	(50.0%)	0.53	(0.19-1.50)
rs2232365	AA	10	(34.5%)	10	(29.4%)	1.00	(Reference)
	AG + GG	19	(65.5%)	24	(70.6%)	0.79	(0.27-2.30)

[a]Statistical analysis of genotype distribution and allele frequencies revealed no significant differences between cases and controls. OR: odds ratio; CI: confidence interval.

The frequency of heterozygotes was slightly decreased in ALL patients in comparison to controls for both polymorphisms. Likewise, the frequency of the variant allele carriers was slightly more frequent in controls than in ALL patients. Allele frequencies and genotype distributions were not statistically different between ALL patients and healthy controls.

We also analyzed the association between the genotype and allele frequencies of the two SNPs studied, related to clinical outcome. In this context, relative risk was evaluated using all ALL patients. There was no association between genotypes and allelic variant carriers for *FOXP3* polymorphisms in relation to ALL clinical outcomes (Table 3 and Table 4).

Table 3. Risk association between *FOXP3* genotypes and ALL clinical outcomes.

SNPs	Groups	Genotypes				RR (95% CI)	*p* value*
		AA		CA+CC			
		n	%	N	%		
rs3761548	Low-Risk	3	17.6	16	82.4	1.67 (0.40-6.92)	0.74
	High-Risk	10	9.4	32	90.6		
		GG		AG+AA			
rs2232365	Low-Risk	6	47.0	13	53.0	1.33 (0.42-4.21)	0.78
	High-Risk	16	62.0	26	38.0		

*Fisher's exact test, p>0.05. RR: relative risk.

Table 4. Risk association between polymorphic allele carriers of *FOXP3* polymorphisms and ALL clinical outcomes.

SNPs	Groups	Allele Carriers				RR (95% CI)	*p* value*
		CC		Allele A carrier			
		n	%	n	%		
rs3761548	Low-Risk	6	31.6	13	68.4	0.75 (0.24-2.37)	0.76
	High-Risk	16	38.1	26	61.9		
		AA		Allele G carrier			
rs2232365	Low-Risk	8	42.1	11	57.9	0.45 (0.15 – 1.35)	0.17
	High-Risk	26	61.9	16	38.1		

*Fisher's exact test, p>0.05. RR: relative risk.

Moreover, we assessed the possible association between ALL susceptibility and the combination of simultaneous presence of two polymorphisms in homozygous (OR 0.88; CI 95% 0.34-2.24) or at least in heterozygous (OR 0.80; CI 95% 0.39-1.67), but also no statistical significance was observed, even when the association was assessed in accordance to clinical outcome subgroups (OR 1.70; CI 95% 0.32-9.08).

Discussion

ALL is caused by acquired genetic abnormalities in hematopoietic precursor cells and it is believed leukemia arises from a single aberrant precursor lymphocyte, which acquires the initiating genetic abnormality. Much speculation has been proposed underlying causal mechanisms of childhood leukemia, although it is well established that most of cancers are the result of combinatorial impacts of crucial exposures, modifying influences, inherited

susceptibility and chance (INABA, H. et al., 2013). Moreover, the clinical and biological aspects of the ALL are well documented, but little is known about individual and populational susceptibility.

The natural history of pediatric leukemia usually involves pre-natal initiation of preleukemic clones (frequently by chromosome translocation) followed by postnatal promotion, secondary mutation and overt disease. Latency after initiation can be very variable (a few months to 15 years) (GREAVES, M., 2006). Therefore, secondary mutations might involve genes related to the immune system tolerance.

In this context, KIM, M. S. et al., (2011) investigated somatic mutations between exons 2-12 of *FOXP3* gene in acute leukemias. Although a few samples were analyzed, they did not find any evidence of mutation, suggesting that there is not involvement of mutations of *FOXP3* gene in leukemogenesis. However, they did not show germinative polymorphism analysis of this gene.

Here we investigate the involvement of two germinative *FOXP3* polymorphisms in ALL susceptibility and clinical risk association. Differences observed in genotype distribution and allele frequencies for both polymorphisms were not associated with ALL in a southern Brazilian population. In addition, we believe that considering separate genotyping for female and male subjects was able to avoid possible conflicting data in a case-control study, once males are hemizygous for this gene.

The heterogeneity of cell types is reflected in the heterogeneity of clinical presentation, prognosis and, possibly, risk factors in ALL (ORSI, L. et al., 2012). Lymphocyte typing reveals two subtypes of ALL malignant cells, B cell (B-ALL) and T cell (T-ALL) (ONCIU, M., 2009). Advances in cellular immunology have made immunophenotype analysis by flow cytometry an essential tool in the diagnosis and classification of ALL, in that it is now far easier to distinguish

the source and differentiation stages of ALL accurately, providing a reference point for clinical treatment (QIU, Y. et al., 2009).

In childhood, the success of chemotherapy protocols has ranged 80% of cure rates (PUI, C. H. et al., 2001; PUI, C. H. et al., 2008). Nonetheless, it depended in part on the recognition of prognostic variables and biological subtypes of the disease, which merited differential treatment (PUI, C. H. et al., 2004).

Tregs are known to play a prominent immunosuppressive role in patients with de novo acute myeloid leukemia (AML) (MAILLOUX, A. W. et al., 2012), and recently it have been confirmed the presence of increased numbers of Tregs in patients with ALL (WU, C. P. et al., 2012). The functional impact of Tregs in the setting of tissue based malignancies such as solid tumors and those based primarily in the blood and bone marrow, such as acute leukemia, may be quite different. However, relatively few studies have addressed either the role of Tregs in primarily leukemic diseases or the utility of Treg quantification in predicting clinical outcome (KELLEY, T. W. e PARKER, C. J., 2010).

The insights into the biology of Treg cells had culminated with the establishment of FOXP3 as a critical transcription factor for specification and maintenance of Treg phenotype such that genetic deficiencies of this marker are associated with development of severe autoimmune diseases (SAKAGUCHI, S. et al., 2010). Moreover, loss of expression and somatic mutation of human *FOXP3* gene were found in human breast and prostate cancers (ZUO, T. et al., 2007a; ZUO, T. et al., 2007b; WANG, L. et al., 2009), suggesting that it may be a tumor suppressor and inactivation of FOXP3 might contribute to human cancer development (KIM, M. S. et al., 2011).

Polymorphisms in the promoter region may potentially alter gene expression by changing the binding specificity of transcription factors to their binding sites and by modifying

the kinetics of transcription initiation (HANEL, S. A. et al., 2011). SHEN, Z. et al., (2010) described that AA genotype of rs3761548 polymorphism of *FOXP3* gene promoter causes the loss of binding with some transcription factors, such as E47 and C-Myb, leading to defective transcription of *FOXP3*.

Our group has been studying common genetic variations in genes related to tumor development and immune system in different cancers. Recently, DE LOURDES PERIM, A. et al., (2013) demonstrated a positive association between CXCL12 (rs1801157) and TP53 (rs1042522) polymorphisms and ALL risk, indicating that CXCL12 and TP53 genes could be potential markers for susceptibility in ALL, either independently but especially when both risk genotypes are inherited simultaneously.

Despite the fact that a lack of immune stimulation was considered a suspected risk factor for childhood ALL and Tregs play an important role in immunosurveilance, the involvement of the master regulator of Tregs, the forkhead box p3 transcription factor (FOXP3) was assessed through the analysis of two polymorphisms rs3761548 and rs2232365 to predict population risk relevant to ALL and did not find association between the presence of the allele nor the homozygous genotypes, when comparing cases and controls. Even when the investigation was directed to clinical outcome, these two polymorphisms were not associated with progression of ALL.

In conclusion, although these polymorphisms may be associated with risk for some diseases, the present work suggests that *FOXP3* gene polymorphisms (rs3761548 and rs2232365) do not affect susceptibility for ALL in southern Brazilian patients.

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Conflict of Interests

The authors declare that they have no conflict of interest.

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ARTIGO 4

DECREASED TGFB1 PLASMA LEVELS AND INFLUENCE OF *FOXP3* GENE POLYMORPHISMS IN BRAZILIAN PATIENTS WITH ACUTE LYMPHOBLASTIC LEUKEMIA

ABSTRACT

Acute lymphoblastic leukemia (ALL) is a malignant disorder that originates from one single hematopoietic precursor committed to the B- or the T-cell lineage. Acquisition by the precursor of a series of genetic abnormalities disturbs its normal maturation process, leading to differentiation arrest and proliferation of the transformed cell. It has been demonstrated the significance of regulatory T cells (Tregs) in carcinogenesis, which acting in the suppression of immune activation as critical mediators of homeostasis and self-tolerance, and are associated with the tumor microenvironment. Their suppressive phenotype are maintained by the expression of Forkhead box P3 (FOXP3) transcription factor and stimulatory cytokines as IL10, IL35 and transforming growth factor beta (TGFB), a molecule known to be pleiotropic. This study investigated the association of rs2232365 and rs3761548 genetic polymorphisms of *FOXP3* promoter region with plasma levels of TGFB1 in ALL patients and healthy controls. We genotyped the *FOXP3* SNPs in 61 ALL patients and 73 controls. The plasma TGFB1 was measured by enzyme-linked immunosorbent assay (ELISA). No evidence was found for an association of the *FOXP3* SNPs and ALL susceptibility, nor recurrence status. Mean TGFB1 plasma levels of ALL were 11.31ng/mL, whereas those of controls were 24.12ng/mL ($p<0.0001$). *FOXP3* rs2232365 and rs3761548 polymorphisms were not associated with TGFB1 plasma levels deviations therein groups, but they were significantly decreased comparing genotypes between ALL and control groups. These results indicates that *FOXP3* rs3761548 and rs2232365 polymorphisms were not related to risk, prognosis nor TGFB1 plasma levels deviations. Nevertheless, downregulation of TGFB1 expression observed in plasma ALL might result in dysregulated proliferation of malignant cells, and it might modulates FOXP3 effects, contributing for acute lymphoblastic leukemia pathogenesis.

KEYWORDS: FOXP3, TGFB1, acute lymphoblastic leukemia, SNP.

INTRODUCTION

Acute lymphoblastic leukemia (ALL) is the most prevalent form of cancer in children, accounting for 72% of all cases of pediatric leukemia (2008; MALONEY, K. W. et al., 2012). The disease arises from genetic alterations that abrogate precursor B- and T-cell differentiation, during hematopoiesis, and drive aberrant cell proliferation and survival (MORICKE, A. et al., 2010), resulting in monoclonal and/or oligoclonal buildup of hematopoietic precursor cells of the lymphoid series within the bone marrow.

Signal transduction pathways that normally control hematopoiesis are disrupted during the pathogenesis of ALL (DONG, M. e BLOBE, G. C., 2006). The transforming growth factor-beta (TGFB) signaling pathway is one such pathway that has a defined role in regulating normal hematopoiesis and is frequently dysregulated in hematologic malignancies (FORTUNEL, N. O. et al., 2000; LARSSON, J. e KARLSSON, S., 2005; RUSCETTI, F. W. et al., 2005).

The effects of TGFB on hematopoietic cells and hematopoiesis are both cell and context specific. The antiproliferative and prodifferentiation effects of TGFB have also been established *in vivo*, with exogenous TGFB1 significantly inhibiting proliferation of granulocyte, erythroid, megakaryocyte, and macrophage progenitor cells (GOEY, H. et al., 1989) and delaying hematologic recovery after treatment of mice with 5-fluorouracil (JANSEN, R. et al., 1991).

In addition, TGFB plays an important role in the generation of regulatory T cells (Tregs) from $CD4^{+}CD25^{-}$ precursors (FU, S. et al., 2004). $CD4^{+}CD25^{+}$ Tregs have been shown to be critical for the maintenance of immunologic tolerance (GRIMMIG, T. et al., 2013). Moreover, the transcription factor forkhead box P3 (FOXP3) has been identified not only as a key player in the immunosuppressive functions of Tregs but also as a definitive marker of $CD4^{+}CD25^{+}$ Tregs (HORI, S. et al., 2003).

Stable *FOXP3* expression is clearly a prerequisite for the maintenance of suppressive properties in Treg cells. The synergistic action of signals downstream of the T-cell receptor (TCR), co stimulatory molecules and cytokine receptors is required for the active transcription of *FOXP3* (HUEHN, J. et al., 2009). In this context, VENUPRASAD, K. et al., (2008) showed that TGFB-inducible early gene 1 (TIEG1; also known as KFL10) can bind to the *FOXP3* promoter and cooperate with itchy E3 ubiquitin protein ligase homologue (ITCH) to induce FOXP3 expression.

In addition, TGFB-induced transcription factor mothers against decapentaplegic homologue 3 (SMAD3) has been shown to control the activity of a newly identified *FOXP3* intronic enhancer element in cooperation with nuclear factor of activated T cells NFAT (TONE, Y. et al., 2008). This indicates that sensitivity to TGFB levels might account for differences in activation-induced FOXP3 expression.

Polymorphisms have been described in various regions of the *FOXP3* gene, such as the promoter, intron and exon regions (ODA, J. M. et al., 2013). Critical for the initiation and regulation of *FOXP3* transcription is its conserved promoter sequence, located upstream of the transcriptional start site (SAKAGUCHI, S. et al., 2008), within two polymorphisms, rs3761548 and rs2232365, were described and associated with risk and prognosis in common multifactorial human diseases (GAO, L. et al., 2010; ZHANG, Y. et al., 2012; JAHAN, P. et al., 2013; YANG, Q. et al., 2013; ZHANG, J. et al., 2013). Although, the relationship between the presence of polymorphic alleles of *FOXP3* and TGFB expression in leukemia patients was not investigated until now.

The proposal of this study was verify the possible implication of two genetic polymorphisms of promoter region of *FOXP3* in TGFB1 plasma levels and susceptibility or recurrence risk status of ALL.

METHODS

Study Population

Following approval from the Human Ethics Committee of the State University of Londrina, Paraná, Brazil (No. 214/09 – CAAE No. 0164.0.268.000-09), inclusion of the individuals to the study was conditioned by an obtained written informed consent form from parents regarding the use of their children and adolescents blood samples for research studies.

Blood samples (5 mL) were collected from sixty one patients with ALL seen in the University Hospital of Londrina and Cancer Institute of Londrina between March of 2009 and November 2012. Seventy-three unrelated children with no evidence of leukemia or other cancer were enrolled as control group. The control subjects did not have a history of hematological disturbances and had no clinical evidences for cancer development. Samples for this experiment were chosen from a southern Brazilian population based cohort study to represent childhood and adolescents from 3 months to 21 years, with ALL and control subjects free of neoplasia.

The ALL diagnostic criteria were based on the guidelines proposed by Hematology Department of the University Hospital. Recurrence risk was evaluated through the GBTLI Protocol (Brazilian Group of Childhood Leukemia Treatment Protocol - 99) which is based on the Cancer Therapy Evaluation Program, proposed by the National Cancer Institute (INCA, 2011). Then, patients were classified into low or high-risk group considering age at diagnosis, leukocyte count, immunophenotyping, involvement of tissues other than bone marrow and responsiveness to treatment.

Procedures of sample processing

Plasma was obtained by centrifugation of heparinized peripheral blood and stored in aliquots at -80°C until analysis. All the analyses were performed on samples that had not been

previously thawed. The time from sample collection to processing was always lower than 24 months. Samples were kept in a freezer connected to an uninterruptible power supply to guarantee the maintenance of optimum temperature.

DNA extraction

Genomic DNA was isolated from 200 µL peripheral blood cells using the DNA Isolation kit Mini Spin Plus (Biopur, Biometrix Diagnostica Ltda, Curitiba, Brazil) according to the manufacturer protocol. After precipitation with ethanol, the pellet was resuspended in 50 µL of Biopur Kit specific buffer and quantified by spectrophotometry for later use in genotyping analyses. Samples were frozen (-20°C) until analysis.

Genotyping

Allele Specific Polymerase Chain Reaction (AS-PCR) analysis was performed to determine the genotype of two polymorphisms of *FOXP3*, as described previously (GAO, L. et al., 2010) with modifications. Amplification was performed in Mastercycler Gradient Thermalcycler (Eppendorf, U.S.A.) with 100ng of genomic DNA, 150pmol of each primer, 100µM total dNTP, 1.5mM $MgCl_2$, 1X PCR Buffer and 1.25 units of Taq DNA polymerase (Invitrogen, Brazil).

The PCR conditions for -3279, C/A (rs3761548) and -924, A/G (rs2232365) were an initial preheating step for 10min at 94°C. Then a subsequent 35 cycles consisted of 94°C for 30sec, 67°C for 30sec, and 72°C for 1min, followed by 1 cycle of 72°C for 10min. The 334bp (A) e 333bp (C) fragments encompassing the C to A (rs3761548) polymorphic site in the promoter region of *FOXP3* gene were amplified using specific primers 5’-CTGGCTCTCTCCCCAACTGA-3’ and 5’-ACAGAGCCCATCATCAGACTCTCTA-3’ for

A allele and 5'-TGGCTCTCTCCCCAACTGC-3' and 5'-ACAGAGCCCATCATCAGACTCTCTA -3' for C allele, whereas the A to G polymorphism (rs2232365) was amplified using specific primers 5'-CCCAGCTCAAGAGACCCCA -3' and 5'-GGGCTAGTGAGGAGGCTATTGTAAC -3' for A allele and 5'-CCAGCTCAAGAGACCCCG-3' and 5'-CTATTGTAACAGTCCTGGCAAGTG-3' for G allele, revealing a 442 bp and 427 bp PCR products, respectively. The amplified PCR products were analyzed by 10% acrylamide gel electrophoresis, visualized after silver nitrate staining.

Determination of plasma TGFB1 levels

Analysis was performed on blood plasma, using ELISA BD OptEIA Set Human TGFB1 (BD Biosciences, San Diego, U.S.A.). In brief, blood plasma from each group was collected, added into the precoated plate with anti-human TGFB1 monoclonal antibody and incubated overnight at 4-8ºC. After, samples were washed and biotinilated secondary antibody-HRP+Sav conjugated were added. After wash, 3,3',5,5'-tetramethylbenzidine (TMB) Peroxidase substrate were immediately added, followed by 1M Phosphoric acid stop solution. The ELISA plate was read at absorbance OD of 450nm wavelength using ELX-300 Reader (BioTek Instruments Inc., VT, U.S.A.). The results were expressed in ng/mL. Each experimental and control sample was assayed in two biological replicates. Data processing were performed using the analytical curve-fitting software Gen5 (BioTek).

Statistical Analysis

Odds ratio (OR) was performed to obtain association study for ALL susceptibility and Relative Risk (RR) for recurrence status (low and high risk), at the 95% confidence intervals (CIs). OR, RR and 95% confidence intervals were assessed by logistic regression using Prism 5 for Windows (GraphPad Software, U.S.A.). Genotype frequencies for *FOXP3*

polymorphisms were tested against Hardy–Weinberg equilibrium by the chi-square test. For each candidate SNP, we followed the analytical approach reported for each locus, assuming at least one of trend (allelic test) and genotypic models. The combined genotypes data were further stratified by subgroups of recurrence risk status (high and low risk). Two-sided tests of statistical significance at the 5% level were performed using the Prism 5 for Windows (GraphPad Software). Univariate analyses were performed to compare the cytokine levels between ALL patients and controls among genotypes using *t* tests (Mann-Whitney U test). In all tests, significance was accepted for p-values of <0.05.

RESULTS

Clinical characteristics

Both ALL and control groups were similar regarding to age (means Control= 11.25± 5.5 years; ALL= 9.65± 6.9 years). In accordance, there were approximately equal numbers of males and females in both groups (control: 53.4% males and 46.6% females vs. ALL: 52.5% males and 47.5% females). Forty-two patients (68.85%) were classified in high recurrence risk group and 19 (31.15%) in low recurrence risk group. Most of our sample, both patients and controls, were predominantly Caucasian, a prevalent population in southern Brazil due to European colonization.

Genotype distribution of *FOXP3* polymorphisms

The controls and the ALL female patients were both in Hardy-Weinberg equilibrium for the two *FOXP3* polymorphisms (rs3761548 p=0.25 and p=0.39; rs2232365 p=0.96 and p=0.37, respectively). The distribution of genotypes and allele frequencies of rs3761548 and rs2232365 is summarized in **Table 1**. *FOXP3* gene is located on X chromosome, thus we performed

separate genotyping for female and male subjects, avoiding possible conflicting data in a case-control study.

For rs3761548 *FOXP3* SNP, the variant hemizygote A genotype was slightly more frequent in ALL male patients than in controls (37.5% and 35.9%, respectively). On the other hand, ALL female patients with AA genotype were almost 2-fold less frequent than females in control group (6.9% vs. 14.7%). The frequencies of heterozygote AG genotypes of rs2232365 were higher both in ALL and control female groups (41.4% vs. 50.0%). In male groups, the hemizygote G genotype was slightly less frequent in ALL than in controls (46.9% vs. 53.8%). However, none of these differences in genotype distribution among groups was statistically significant (Table 1).

Table 1. *FOXP3* genotypes among ALL and control subjects, and their associations with ALL risk.

Gene (gender)	SNP ID	Genotype	ALL		Control		OR (95% CI)	p[a]
			N°.	%	N°.	%		
FOXP3 (males)	rs3761548	C	20	62.5	25	64.1	1.1 (0.41-2.80)	1.00
		A	12	37.5	14	35.9		
	rs2232365	A	17	53.1	18	46.2	1.3 (0.52-3.40)	0.63
		G	15	46.9	21	53.8		
FOXP3 (females)	rs3761548	CC	19	65.5	17	50.0	1 (reference)	
		CA	8	27.6	12	35.3	0.60 (0.20-1.80)	0.41
		AA	2	6.9	5	14.7	0.36 (0.06-2.10)	0.41
	rs2232365	AA	10	34.5	10	29.4	1 (reference)	
		AG	12	41.4	17	50.0	0.71 (0.22-2.20)	0.57
		GG	7	24.1	7	20.6	1.00 (0.26-3.90)	1.00

ALL = acute lymphoblastic leukemia. OR= Odds ratio. CI= confidence interval.
[a]p values from unconditional logistic regression analyses, *Fisher* exact test.

The association of *FOXP3* SNPs and recurrence status in ALL patients was assessed in view of possible influence of the variant genotypes. In this context, we evaluated the relative risk using all ALL patients. There was no association between genotypes of rs3761548

(RR=1.67, 95% CI=0.40-6.92) and rs2232365 (RR=1.33, 95% CI=0.42-4.21) and allelic variant carriers for rs3761548 (A) (RR=0.75, 95% CI=0.24-2.37) and rs2232365 (G) (RR=0.45, 95% CI=0.15-1.35) in relation to ALL recurrence status.

Analysis of TGFB1 Plasma Levels

Determination of TGFB1 plasma levels was performed in all ALL patients and control subjects, and results are shown as mean ± SEM. The mean TGFB1 concentration for ALL patients (11.31 ng/mL± 1.7) was significantly lower than that among the control group (24.12 ng/mL± 1.1; p=0.0007) (**Figure 1**).

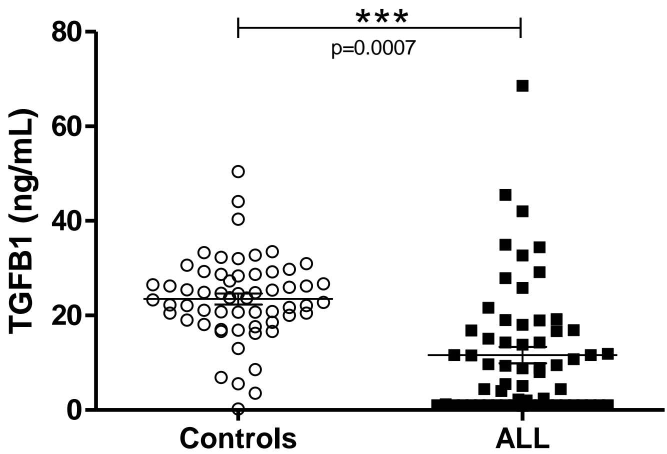

Figure 1. TGFB1 plasma concentrations in ALL patients and controls. Plasma TGFB1 levels obtained from 73 controls and 61 ALL patients. Groups were compared by the Student *t* test. The horizontal bars represent the mean values; S.E.M. as error bars

We compared the plasma TGFB1 levels in females with the levels in males for both evaluated groups. There were no significant differences between females and males for ALL patients and controls. In addition, ALL females showed significantly lower plasma levels of TGFB1 than control females (8.33ng/mL± 1.77 vs 22.12ng/mL± 1.84; p=0.0001).

Plasma TGFB1 concentration were associated with *FOXP3* genotypes using all ALL patients and controls. Regarding to *FOXP3* rs3761548, homozygous for the variant allele (A) showed higher TGFB1 levels in ALL patients (16.70ng/mL; p>0.05) and controls (28.33ng/mL;

p=0.01) against homozygous carriers for common allele (C) (ALL CC 11.48ng/mL and Controls CC 20.87ng/mL). In control group, there was significant increase of TGFB1 plasma levels in AA genotype (28.33ng/mL; p=0.01) and allele A carriers (28.03ng/mL; p=0.02) of *FOXP3* rs3761548 polymorphism comparing to CC genotype (20.85ng/mL).

Likewise, mean TGFB1 levels were significantly decreased in ALL patients, comparing their genotypes to those of controls (CC 11.0ng/mL vs 21.0ng/mL; CA 4.5ng/mL vs 28.5ng/mL; AA 16.0ng/mL vs 28.0ng/mL) (**Figure 2a**).

Correspondingly, ALL patients presented reduced TGFB1 plasma levels in the comparison of *FOXP3* rs2232365 genotypes to the controls (AA 12.4ng/mL vs 22.87ng/mL; AG 7.32ng/mL 25.72ng/mL; GG 13.01ng/mL vs 24.12ng/mL) (**Figure 2b**).

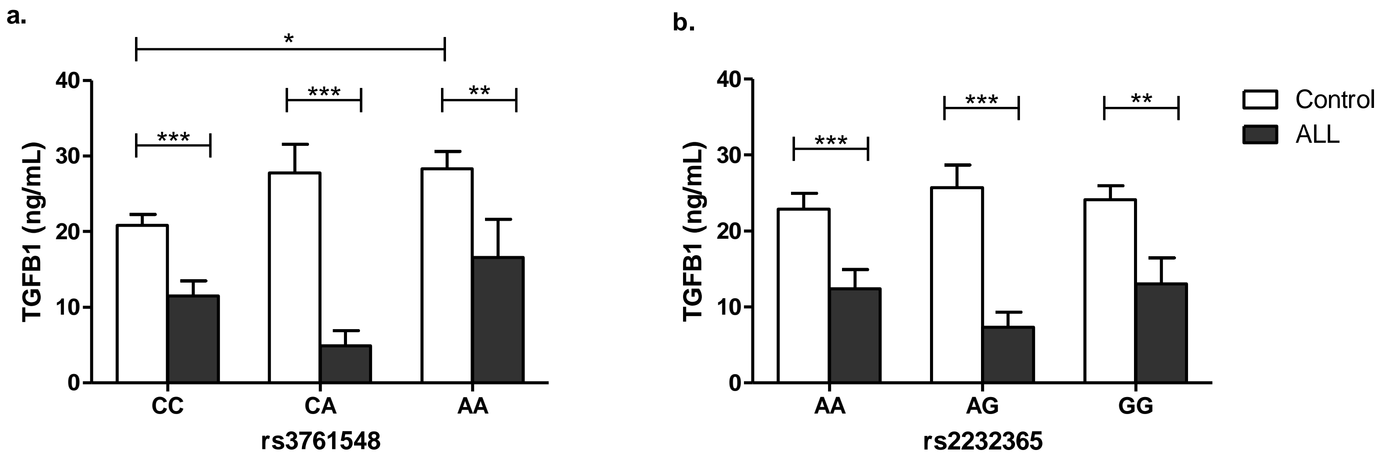

Figure 2. TGFB1 plasma concentrations and *FOXP3* genotypes in ALL patients and controls. * p<0.05; ** p<0.01; *** p<0.001, Mann-Whitney U test.

Furthermore, lower plasma levels of TGFB1 were detected in carriers for both variant homozygous genotypes (rs3761548 AA and rs2232365 GG) (ALL= 18.26ng/mL vs Controls= 26.96ng/mL). Although there was a tendency, the observed differences were not significant (p=0.051).

To study whether the observed differences for the plasma levels of TGFB1 among *FOXP3* genotypes might be explained by a general difference between females and males, we

analyzed the plasma levels of this cytokine independently, since males are hemizygous for this gene. No gender differences were observed for TGFB1 plasma levels (*p* values ranging from 0.11 to 0.81) among *FOXP3* genotypes, showing that the observed differences in cytokine levels between ALL patients and controls are not due to general differences in gender.

Considering recurrence status of ALL patients, slightly increased TGFB1 plasma levels were found in high risk comparing to low risk patients (12.19ng/mL± 2.11 vs. 10.36ng/mL± 3.06; p=0.38). However, these differences in plasma levels among recurrence status subgroups were not statistically significant.

DISCUSSION

It has become reasonable that Tregs may be involved in tumor development and progression, since they have been evidenced in distinct types of cancer, on occasion in tumor microenvironment or at peripheral blood. As a master regulator of Treg function, expression of *FOXP3* must be accompanied by stimulatory cytokine signals, which will define T cell fate. In this context, TGFB seemed not to be sufficient for imprinting T cells with the permanent expression of *FOXP3* that is needed for a stable Treg cell phenotype (HUEHN, J. et al., 2009).

MARUYAMA, T. et al., (2011) reviewed the progress in understanding the complex molecular events that drive *FOXP3* gene expression and allow functional regulatory T cells to develop. They discussed the transcription factor networks at play in the controlled expression of *FOXP3* and the generation of regulatory T cells. How TGFB promotes *FOXP3* expression remains an area of intense investigation, with a number of factors shown to bind the *FOXP3* locus in response to a TGFB signal.

In this study, two polymorphisms of *FOXP3* gene promoter region were assessed in view of their implication on TGFB1 plasma levels in ALL patients and controls. Firstly, we investigate whether two promoter region polymorphisms of *FOXP3* could be associated with

ALL susceptibility or recurrence status classification. Polymorphisms in the promoter region may potentially alter gene expression by changing the binding specificity of transcription factors to their binding sites and by modifying the kinetics of transcription initiation (HANEL, S. A. et al., 2011).

Even so, there was no association between alleles or genotypes of rs3761548 and rs2232365 *FOXP3* polymorphisms in promoter region, suggesting that there is not involvement of these polymorphisms in ALL susceptibility nor recurrence risk status (Table 1).

It is known that tumor cells are often resistant to signals that inhibit growth of their normal cell counterparts. Escape from such control mechanisms may contribute to malignant transformation (LOTZ, M. et al., 1994). Nevertheless, TGFB is an essential regulator of cellular processes, including proliferation, differentiation, migration and cell survival (DONG, M. e BLOBE, G. C., 2006).

In carcinogenesis, TGFB has a bifunctional role retarding tumor development but enhancing progression once neoplastic transformation has occurred and the growth inhibitory response to TGFB has been lost (MOSES, H. L. et al., 1994). Further, TGFB regulates leukemia cells proliferation and is perhaps the most potent endogenous negative regulator of hematopoiesis (DOSEN-DAHL, G. et al., 2008).

MUROHASHI, I. et al., (1995) described differential effects of TGFB1 on normal and leukemia hematopoietic human cell proliferation. Leukocytes of controls and patients with hematologic malignancies were treated with TGFB1 and different growth factors. They demonstrated that TGFB1 treatment inhibited mRNA expression in acute myeloid leukemia blasts for c-myc and/or c-myb, promoting growth suppression.

In this study, we found significantly diminished plasma concentrations of TGFB1 in ALL patients compared with those in control subjects (p=0.0007). These results are in accordance to the findings of CHEN, Y. et al., (1998), who shown that serum TGFB1 levels

were significantly decreased in acute leukemia patients, restored to normal in the patients achieved complete remission, and tended to decrease again in the recurrent patients.

The signaling in which TGFB controls cell proliferation is not well understood. Indeed, following the TGFB induction of the TGFBR-I/TGFBR-II/Smad2/3/4 signaling cascade, the activated Smad proteins (Smad 2/3/4 complexes) target the promoters of c-myc and cyclin-dependent kinase (CDK) genes, and repress their transcription in cooperation with nuclear co-repressors (MOUSTAKAS, A. et al., 2002; DERYNCK, R. e ZHANG, Y. E., 2003; HUANG, S. S. e HUANG, J. S., 2005). Moreover, many lines of evidence indicate that other signaling pathways, in addition to the prominent TGFBR-I/TGFBR-II/Smad2/3/4 signaling cascade, are required for mediating TGFB-induced growth inhibition (HUANG, S. S. e HUANG, J. S., 2005).

It is known that in children T-cell acute lymphoblastic leukemia (ALL), Smad3 protein is absent or significantly decreased, however Smad3 mRNA is present in T-cell ALL and normal T-cells at similar level. The level of Smad3 is decisive for the T-cell response to TGFB. A reduction in Smad3 interplays with other oncogenic events, such as alterations in retinoblastoma pathway, to precede T-cell leukemogenesis (WOLFRAIM, L. A. et al., 2004).

Thus, we suggested that downregulation of TGFB1 expression observed in plasma ALL patients might result in unlimited proliferation of lymphoid malignant cells, which in turn may contributes to the bone marrow hyperplasia and peripheral blood invasion of leukemic clones.

It has been reported that alignment of the *FOXP3* locus from different species has evidenced the presence of several highly conserved regions. In addition to the promoter, these include three noncoding regions that could play a role in regulation of the *FOXP3* gene (MANTEL, P. Y. et al., 2006; ZHENG, Y. et al., 2010). They were described as conserved non-coding sequences (CNS) 1, 2 and 3. Although often referred to as enhancers, detailed analysis

of these regions has been undertaken and transcription factors have been shown to bind and influence *FOXP3* gene induction box (MARUYAMA, T. et al., 2011).

Furthermore, there are intense current data demonstrating that TGFB can drive *FOXP3* expression, although detailed pathway has not been determined. Following TGFB stimulation of T cells, Smad3 has been shown to rapidly bind the CNS1, suggesting a role for this transcription factor in *FOXP3* expression (MARUYAMA, T. et al., 2011).

Another factor downstream of TGFB and involved in *FOXP3* induction is TGFB inducible early gene 1 product (TIEG1), a transcription factor rapidly induced upon TGFB stimulation. Ubiquitination of TIEG1 by the E3 ubiquitin ligase Itch promotes transcriptional activation of the *FOXP3* promoter and FOXP3 expression (VENUPRASAD, K. et al., 2008).

Also, DOSEN-DAHL, G. et al., (2008) have demonstrated TIEG1 expression in both normal B progenitor cells and ALL cells, which increased rapidly upon TGFB and bone morphogenetic protein 6 (BMP-6) treatments. Stimulation with TGFB or BMP-6, as well as overexpression of TIEG1 inhibited proliferation, and *in vitro*, interaction with stroma cells induced TIEG1 expression in ALL cells, abrogated their proliferation and protected cells against chemotherapeutic treatment with cytosine arabinoside, an analogue of the nucleoside deoxycytidine, used in consolidation therapy of ALL (ESTLIN, E. J. et al., 2001).

Altogether, despite the fact that Smad3 acts cooperatively with NFAT in the activation of *FOXP3* transcription (TONE, Y. et al., 2008), TIEG1 and NFAT were not investigated in this context, our results strengthened the hypothesis that downstream effects of TGFB signaling cascade are dysregulated in ALL, since TGFB1 levels are reduced, and it might modulates FOXP3 effects on leukemic cells.

Genetic polymorphisms of *FOXP3* have been characterized in translated and untranslated regions (ODA, J. M. et al., 2013). According to HOOGENDOORN, B. et al., (2003), promoters are involved in initiating transcription and are therefore among the many

important *cis*-acting elements that regulate gene expression that might harbor functionally relevant polymorphisms.

In this study, *FOXP3* rs3761548 homozygous for the variant allele (AA) showed higher TGFB1 levels in ALL patients and controls against homozygous carriers for common allele (CC). Decreased plasma TGFB1 levels were found in ALL patients, comparing their genotypes to those of controls (Figure 2a). Further, ALL patients presented reduced TGFB1 plasma levels in the comparison of *FOXP3* rs2232365 genotypes to the controls (Figure 2b).

In conclusion, mean TGFB1 levels among *FOXP3* genotypes were significantly diminished comparing ALL patients and controls, and this decrease in plasma level of TGFB1 was found to be related to the presence of common alleles (CC for rs3761548 and AA for rs2232365) of *FOXP3* polymorphisms. Since TGFB is closely related to the activation of the *FOXP3* transcription, we hypothesized that TGFB1 concentration in circulating plasma might impair FOXP3 effects in the presence of genetic polymorphisms in the promoter region and TGFB1 might be considered a serological biomarker for ALL.

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4 CONSIDERAÇÕES FINAIS

- O polimorfismo rs333 (delta32) do gene *CCR5* não foi detectado em homozigose nas amostras dos grupos controle e leucêmico. Ainda, a análise dos genótipos não demonstrou diferenças significativas na frequência do genótipo CCR5/delta32 entre os grupos controle e leucêmico, sugerindo ausência de efeito deste polimorfismo na susceptibilidade ao desenvolvimento da LLA e ao prognóstico em relação ao status de risco da doença.
- A análise dos alelos e genótipos dos polimorfismos do gene *FOXP3* nos grupos controle e leucêmico demonstrou, pela primeira vez na literatura, que não houve associação com o risco de desenvolvimento da LLA nem com os parâmetros clínicos (alto e baixo risco) dos pacientes, e portanto não foram considerados fatores de susceptibilidade genética para a LLA na população sul-brasileira.
- A expressão proteica do TGFB1 no plasma de pacientes com LLA foi significativamente reduzida em relação ao grupo controle livre de neoplasia.
- Os níveis plasmáticos de TGFB1 diferiram significativamente entre os genótipos dos polimorfismos rs3761548 e rs2232365 do gene *FOXP3*, tanto no grupo controle quanto no grupo leucêmico.
- Portadores dos alelos variáveis (AA para rs3761548 e GG para rs2232365) dos polimorfismos genéticos do *FOXP3* apresentaram níveis maiores de TGFB1 plasmático nos grupos controles e leucêmicos, embora diferença significativa tenha sido observada apenas para o polimorfismo rs3761548, no grupo controle.
- A concentração plasmática reduzida de TGFB1 em pacientes portadores de LLA pode resultar na proliferação incessante de células malignas linfoides, o que poderia contribuir para a hiperplasia medular e a invasão periférica de clones leucêmicos.

- O presente estudo reforça a hipótese da desregulação dos efeitos a jusante da cascata de sinalização ativada pelo TGFB na LLA, uma vez que os níveis de TGFB1 estavam reduzidos, e isto poderia modular os efeitos do FOXP3 nas células leucêmicas.
- Finalmente, o presente trabalho sugere que os polimorfismos genéticos da região promotora do *FOXP3* e a concentração plasmática do TGFB1 estão associados à patogênese da LLA.