PATRÍCIA MIDORI MUROBUSHI OZAWA

AVALIAÇÃO DE POLIMORFISMOS GENÉTICOS DO FATOR DE TRANSCRIÇÃO *FOXP3* E DA QUIMIOCINA *CXCL12* E ANÁLISE IMUNOHISTOQUÍMICA DO FOXP3 E DO RECEPTOR DE QUIMIOCINA CXCR4 EM PACIENTES PEDIÁTRICOS PORTADORES DE MEDULOBLASTOMA E TUMOR DE WILMS

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

Orientador: Profª Dra. Maria Angelica Ehara Watanabe
Co-orientador: Dra. Carolina Batista Ariza

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Londrina, 28 de novembro de 2014.

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"QUE OS VOSSOS ESFORÇOS DESAFIEM AS IMPOSSIBILIDADES, LEMBRAI-VOS DE QUE AS GRANDES COISAS DO HOMEM FORAM CONQUISTADAS DO QUE PARECIA IMPOSSÍVEL".

(CHARLES CHAPLIN)

OZAWA, Patrícia Midori Muroboshi. **Avaliação de polimorfismos genéticos do fator de transcrição FOXP3 e da quimiocina CXCL12 e análise imunohistoquímica do FOXP3 e do receptor de quimiocina CXCR4 em pacientes pediátricos portadores de Meduloblastoma e Tumor de Wilms**. 2014. 80 f. Dissertação (Mestrado em Patologia Experimental) – Universidade Estadual de Londrina, Londrina, PR

RESUMO

O meduloblastoma (MB) é o tumor infantil de sistema nervoso central (SNC) mais comum, sendo responsável por aproximadamente 20 a 25% dos casos nesta faixa etária. Os tumores renais representam de 5 a 10% de todas as neoplasias infantis e destes, 95% são do tipo embrionário, denominado de tumor de Wilms (TW). A quimiocina CXCL12 e seu receptor CXCR4 estão envolvidos em diversos processos biológicos, incluindo o desenvolvimento de vários órgãos, como cerebelo e rim, e até mesmo com o câncer. O fator de transcrição FOXP3 é expresso em diversos tipos celulares, inclusive em células tumorais, e sua expressão parece estar relacionada com o prognóstico dos pacientes. Polimorfismos genéticos presentes em regiões regulatórias de genes como o CXCL12 e FOXP3 parecem ter envolvimento com diversas doenças, incluindo o câncer. Dessa forma, o objetivo deste trabalho foi avaliar a associação de polimorfismos genéticos rs2232365 e rs3761548 e a expressão proteica do FOXP3, bem como o polimorfismo genético rs1801157 do CXCL12 e a expressão proteica do CXCR4 em pacientes diagnosticados com MB e TW. O estudo epidemiológico revelou as seguintes frequências para estes tumores sólidos infantis: TW (43,182%), NB (39,773%), ACT (10,227%) e MB (6,818%). Foi demonstrado que pacientes diagnosticados com MB e com TW possuem expressão citoplasmática de FOXP3 e CXCR4, embora sem correlação com os outros parâmetros analisados (idade, gênero e tamanho de tumor). Foi observada associação entre a presença do alelo A, para o polimorfismo rs1801157 do CXCL12 (OR=5,261; 95% IC: 2,156 a 12,84; p=0,0002), bem como a presença do alelo G, para o polimorfismo rs2232365 do FOXP3 (OR=0,1304; 95% IC: 0,05013 a 0,3394; p<0,0001) com a susceptibilidade ao TW. Sendo assim, é possível afirmar que estes genes podem ser marcadores moleculares promissores de susceptibilidade ao TW, e que embora a expressão proteica de FOXP3 e CXCR4 estejam presentes no MB, mais estudos serão necessários para a elucidação do envolvimento deles com o desenvolvimento e o prognóstico do TW e MB.

Palavras-chaves: Tumor de Wilms. Meduloblastoma. Polimorfismos genéticos. Expressão proteica. FOXP3. CXCL12. CXCR4.

OZAWA, Patrícia Midori Muroboshi. **Genetic polymorphisms analysis of FOXP3 transcription factor and chemokine CXCL12 and immunohistochemistry analysis of FOXP3 and CXCR4 chemokine receptor in childhood patients with Meduloblastoma and Wilms' Tumor.** 2014. 80 p. Dissertation (Master's degree in Experimental Pathology) - State University of Londrina, Londrina, 2014.

ABSTRACT

Meduloblastoma (MB) is the most common childhood tumor of the central nervous system (CNS), accounting for approximately 20 a 25% of all cases in this age. The renal tumors represents 5 to 10% of childhood neoplasias and among them, 95% are characterized as embrionary and it is named Wilms' tumor (WT). The CXCL12 chemokine and its receptor CXCR4 are involved in several biological processes, including the development of several organs like cerebellum and kidney, and even in cancer. The transcription factor FOXP3 is expressed in many cell types, including tumor cells, and its expression appears to be related with patient's prognosis. Genetic polymorphisms present at regulatory regions of genes like CXCL12 and FOXP3 appear to have involvement with several diseases, including cancer. Thereby, the aim of this study was to evaluate the association among FOXP3 genetic polymorphisms rs2232365 e rs3761548 and also its protein expression, as well as the CXCL12 genetic polymorphism rs1801157 and protein expression of its receptor CXCR4 in patients diagnosed with MB and WT. The epidemiologic study showed the following frequencies for these childhood solid tumors: WT (43.182%), NB (39.773%), ACT (10.227%) e MB (6.818%). It was demonstrated that patients diagnosed with MB and WT possess cytoplasmic expression of FOXP3 and CXCR4, although it did not correlate with any parameter analyzed (age, gender and tumor size). It was observed an association for allele A carrier of CXCL12 rs1801157 (OR=5.261; 95% CI: 2.156 to 12.84; p=0.0002), as well as allele G carrier of FOXP3 polymorphisms rs2232365 (OR=0.1304; 95% CI: 0.05013 to 0.3394; p<0.0001), with WT susceptibility. Thus, it is possible to affirm that these genes can be promisor molecular markers to WT susceptibility, and although the protein FOXP3 and CXCR4 are expressed in MB, more studies are needed to elucidate their involvement with WT and MB development and prognosis.

Keywords: Wilms' Tumor. Medulloblastoma. Genetic polymorphisms. Protein expression. FOXP3. CXCL12. CXCR4.

LISTA DE TABELAS

LISTA DE ABREVIATURAS E SIGLAS

%	Porcentagem
°C	Grau Celsius
µg	Micrograma
µL	Microlitro
3'UTR	*Three Prime Untranslated region/* Região não traduzida 3'
ACT	*Adrenocortical tumors/* Tumor adrenocortical
$AgNO_3$	Nitrato de prata
CD4	*Cluster of differentiation 4*/ Grupo de diferenciação 4
CNS	*Central Nervous System/* Sistema nervoso central
CTNNB1	*Catenin (Cadherin-Associated Protein), Beta 1/* Beta 1 catenina (Proteína associada a caderina)
CXCL12	*Chemokine (C-X-C motif) ligand 12/* Ligante 12 de quimiocina C-X-C
CXCR4	*Chemokine (C-X-C motif) receptor 4*/ Receptor 4 de quimiocina C-X-C
DNA	*Desoxyribonucleic Acid/* Ácido desoxirribonucleico
dNTP	*Deoxyribonucleotide triphosphates/* Desoxiribonucleotídeo trifosfato
EGL	*External Granule Cell Layer/* Camada de células granulares externa
FOXP3	*Forkhead box protein 3*/ Proteína *Forkhead box* 3
GPCRs	*G Protein-Coupled Receptors/* Receptores acoplados à proteína G
GRK2	*G Protein-Coupled Receptor Kinase 2/* Receptor de quinase 2 acoplado à proteína G
GRK6	*G Protein-Coupled Receptor Kinase 6/* Receptor de quinase 6 acoplado à proteína G
HER2	*Human Epidermal growth factor receptor 2* / Receptor 2 do fator de crescimento humano epidérmico
IGL	*Internal Granule Cell Layer/* Camada de células granulares interna
INCA	Instituto Nacional do Câncer
MB	*Medulloblastoma*/ Meduloblastoma
mg	Miligrama
min	Minutos
mL	Mililitro
mM	Milimolar

MspI	enzima de restrição extraída da *Moraxella* sp
MYC	*Avian Myelocytomatosis Viral Oncogene Homolog/* Oncogene viral homólogo a mielocitomatose aviária
ng	nanograma
nm	Nanômetros
pb	Pares de base
PCR	*Polymerase Chain Reaction*/ Reação em Cadeia da Polimerase
RFLP	*Restriction Fragment Length Polymorphism/* Polimorfismo no comprimento de fragmentos de restrição
SDF-1	*Stroma derived factor 1/* Fator 1 derivado do estroma
SHH	*Sonic Hedgehog*
SNC	Sistema Nervoso Central
TAE	Tampão Tris-acetato-EDTA
TP53	*Tumor protein P53/* Proteína tumoral P53
Treg	*Regulatory T cell*/ Célula T reguladora
TW	Tumor de Wilms
URL	*Upper Rhombic Lip*/ Lábio Rômbico superior
V	Volts
WHO	*World Health Organization/* Organização Mundial da Saúde
WNT	*Wingless*
WT	*Wilms' Tumor/* Tumor de Wilms
WT1	*Wilms' tumor 1*
WT2	*Wilms' tumor 2*

SUMÁRIO

1 INTRODUÇÃO

1.1. TUMORES PEDIÁTRICOS

O câncer é considerado uma doença genética, e para o seu desenvolvimento é necessário que haja uma sequência de eventos que culminarão na transformação da célula normal em maligna. Existem diversos fatores de risco que influenciam essa transformação, como os genéticos e ambientais. No entanto, no que se refere aos tumores pediátricos, o fator de risco que parece ter maior importância é o genético, uma vez que a idade precoce do diagnóstico sugere exposição baixa aos outros fatores de risco (DAVIDOFF, 2012).

O câncer infanto-juvenil é considerado raro e caracteriza-se por acometer indivíduos de até 19 anos, correspondendo entre 2% e 3% de todos os tumores malignos. O Instituto Nacional de Câncer (INCA) estima para o ano de 2014 cerca de 11.840 novos casos de câncer em crianças e adolescentes até os 19 anos. Considerando-se que a primeira causa de mortalidade dessa faixa etária refere-se às causas externas, como acidentes e violência, a mortalidade por câncer é, atualmente, a primeira causa de mortes por doença nesta população (INCA, 2014).

Existem diversas diferenças entre o câncer que acomete adultos e o que acomete indivíduos na faixa etária até os 19 anos, como por exemplo, diferenças nos locais primários, nas origens histológicas e nos comportamentos clínicos, o que sugere que estes tipos de neoplasias malignas devam ser estudadas de forma diferenciada daquelas originadas em adultos (MALKIN, 1997).

Um dos grandes problemas dos tumores pediátricos é o fato de a sua localização ser em tecidos que estão em desenvolvimento. Uma vez que o tratamento afeta células em proliferação, o desenvolvimento desses órgãos imaturos pode ser seriamente afetado. Este problema pode ser mais grave em pacientes com câncer no sistema nervoso central (SNC), cujo tratamento pode levar ao comprometimento do desenvolvimento cognitivo da criança (DUFFNER, COHEN & PARKER, 1988; JANNOUN & BLOOM, 1990).

Dessa forma, com o melhor entendimento dos mecanismos envolvidos no surgimento desses tipos de câncer é possível que se crie estratégias terapêuticas mais específicas e que gerem menos impacto no desenvolvimento dos pacientes.

1.1.1 MEDULOBLASTOMA

Apesar de o cerebelo ser uma das primeiras estruturas do SNC a se diferenciar, ele atinge a sua configuração madura após aproximadamente 18 meses depois do nascimento. Esse período de formação mais prolongado (em comparação as outras estruturas do SNC) deixa o cerebelo especialmente vulnerável ao desenvolvimento de irregularidades. Tais anormalidades podem se manifestar sob defeitos estruturais óbvios, como as malformações de Chiari e Dandy-Walker, ou então sob neoplasias cerebelares decorrentes do descontrole da diferenciação e proliferação celular, como o meduloblastoma (MB) (CHIZHIKOV & MILLEN, 2003).

O MB é um tumor embrionário que se desenvolve a partir de células transformadas do cerebelo, sendo considerado o tumor cerebral maligno mais comum na infância, correspondendo a aproximadamente 20% dos tumores de SNC nessa faixa etária (OSTROM *et al.*, 2013). A idade aproximada ao diagnóstico é de 6 anos de idade (FARWELL, DOHRMANN & FLANNERY, 1984). Embora o MB seja cerca de dez vezes mais frequente em indivíduos dessa faixa etária quando comparado com os adultos, estes também podem desenvolver MB, embora seja mais raro (SMOLL & DRUMMOND, 2012).

A Organização Mundial da Saúde (OMS) classifica o MB histologicamente em cinco subtipos: clássico, desmoplásico, extrema modularidade, anaplásico e de células gigantes (LOUIS *et al.*, 2007). Recentemente surgiu uma classificação molecular baseada no perfil de expressão gênica desses tumores, que discrimina as vias de sinalização que estão desreguladas no MB, incluindo alterações nas vias WNT (*Wingless*), SHH (*Sonic Hedgehog)* e MYC (*Avian Myelocytomatosis Viral Oncogene Homolog)* (KOOL *et al.*, 2008; NORTHCOTT *et al.*, 2011).

O subtipo clássico apresenta células pequenas e uniformes que possuem uma alta razão núcleo/citoplasma (GILBERTSON & ELLISON, 2008) e é considerado o subtipo mais frequente, juntamente com o desmoplásico (GIANGASPARO *et al.*, 2000). O subtipo desmoplásico apresenta áreas densas, de alta proliferação e ricas em reticulina que circundam nódulos livres de reticulina (KATSETOS *et al.*, 1989). Sabe-se que o subtipo desmoplásico está associado a uma sobrevida maior e que os tumores anaplásicos de células gigantes são os que possuem uma sobrevida inferior, quando comparados ao subtipo clássico (HATTEN & ROUSSEL, 2011).

O MB de extrema nodularidade é caracterizado por nódulos grandes e de formato irregular, diferenciação neurocítica internodular pronunciada e regiões internodulares esparsas

(GIANGASPERO *et al.*, 1999; MCMANAMY *et al.*, 2007). O MB do tipo anaplásico é caracterizado por um pleomorfismo nuclear marcante e alta atividade mitótica, com atipia celular pronunciada e generalizada. O MB de células gigantes apresenta células esféricas com núcleo circular, cromatina aberta e nucléolo central e proeminente, e muitas vezes possui sobreposição citológica com a variante anaplásica, muitas vezes sendo considerado um subgrupo apenas (LOUIS *et al.*, 2007).

O estadiamento dos pacientes é realizado baseado na idade e no estágio da doença. Pacientes com até 3 - 5 anos são considerados infantes, aqueles até 18 anos são considerados crianças e até 21 anos, adultos jovens. A classificação do estágio da doença é feita de acordo com o sistema de estadiamento de Chang (CHANG, HOUSEPIAN & HERBERT, 1969) (Tabela 1) .

Tabela 1. Sistema de estadiamento para metástase de Chang.

Estadiamento M
M0: sem evidência de metástase subaracnóide ou hematogênica
M1: células tumorais microscopicamente encontradas no fluido cerebro-espinal
M2: implante nodular intracraniano além do local primário (no espaço subaracnóide cerebelar/cerebral ou no terceiro ventrículo ou ventrículo lateral
M3: implante nodular no espaço subaracnóide espinal
M4: metástase fora do eixo cerebro-espinal

Os pacientes considerados risco padrão são aqueles com mais de 3 anos, sem evidência de metástase (M0), sem anaplasia e com uma ressecção cirúrgica quase completa do tumor, apresentando um resquício tumoral menor do que 1,5 mm^2. Os pacientes que se encaixam nestes critérios possuem uma sobrevida de até 85% (ZELTZER *et al.*, 1999; PACKER *et al.*, 2006) e correspondem a um terço dos pacientes (ZELTZER *et al.*, 1999). Aqueles que não se encaixam nestes critérios são considerados de alto risco, sendo tratados com uma abordagem mais intensificada, possuindo uma sobrevida de aproximadamente 60% (TARBELL *et al.*, 2013).

Embora a etiologia dos tumores de SNC seja amplamente desconhecida, a investigação genética desses tipos de tumores pode revelar informações importantes sobre

genes que regulam a proliferação, diferenciação, angiogênese, migração e apoptose no sistema nervoso central. Estes estudos podem identificar genes candidatos para terapia gênica direta em uma tentativa de aumentar as chances de cura para os subgrupos de tumores que apresentam prognóstico ruim.

1.1.2 TUMOR DE WILMS

Os tumores renais representam aproximadamente 6% de todas as neoplasias infantis (RIES *et al.*, 2004). Destes, 95% são do tipo embrionário, denominado de nefroblastoma ou Tumor de Wilms (TW). A incidência do TW é de 1 em cada dez mil crianças, especialmente abaixo dos 6 anos de idade (média 3,5) e é igualmente distribuída em relação ao sexo. O TW é na maioria das vezes unilateral e esporádico, porém 5 a 10% das crianças com este tipo de tumor têm envolvimento bilateral, sendo que apenas 1% é considerado hereditário (BRESLOW *et al.*, 2006).

O TW é classificado histologicamente em dois tipos: de prognóstico favorável e desfavorável (BECKWITH & PALMER, 1978). O grupo dos desfavoráveis corresponde a 5% de todos os TW e incluem aqueles tumores que apresentam anaplasia, acometendo principalmente crianças por volta dos 5 anos de idade (GREEN *et al.*, 1994).

Uma das primeiras pistas de problemas genéticos relacionados ao TW surgiu de investigações citogenéticas que mostraram alteração no gene *WT1* (*Wilms' tumor gene 1*) em pacientes portadores de uma síndrome rara, caracterizada por presença de TW, aniridia, anormalidades geniturinárias ou gonadoblastoma e retardo mental (síndrome WAGR) (CALL *et al.*, 1990). Além disso, estudos mostraram alterações numéricas, na maioria das vezes trissomias dos cromossomos 7, 8 e 12 (HÖGLUND *et al.*, 2004).

O gene *WT1* codifica um fator de transcrição que pode agir como gene supressor de tumor. Cerca de 5% dos pacientes diagnosticados com TW esporádico parecem ter mutação no *WT1*. A função de *WT*2 (*Wilms' tumor gene 2*) ainda é desconhecida. Alguns casos de familiares de TW não associados a alterações genéticas dos genes *WT1* e *WT2* sugerem que pode haver outro *locus* relacionado ao aparecimento do tumor de Wilms (SUGIYAMA, 2010). Além das mutações nestes genes, foram identificadas mutações nos genes *CTNNB1* e *TP53* e sabe-se que a mutação no gene *TP53* está presente na variante anaplásica do TW, e a um mau prognóstico (MASCHIETTO *et al.*, 2014).

A taxa de sobrevida de pacientes tratados com uma abordagem terapêutica multimodal tem alcançado 90%, no entanto pacientes que possuem prognóstico ruim, como aqueles com

recidiva ou com presença de metástase, possuem uma taxa de sobrevida menor que não alcança 50% (SMITH *et al.*, 2010). Dessa forma, estudos que objetivem a caracterização molecular do TW são importantes em vista do refinamento do tratamento, uma vez que a identificação de características genéticas podem levar a estratificação em subgrupos em que os pacientes possam ser tratados de acordo com o seu risco de recidiva (DAVIDOFF, 2012).

O microambiente tumoral é de suma importância para o estudo da patogênese do câncer, uma vez que há interação entre as células malignas neoplásicas, o estroma circundante e o sistema imune, tanto inato quanto adaptativo. Essa complexa interação presente no microambiente tumoral influencia a forma como o organismo irá combater o tumor, bem como afeta o crescimento, o potencial invasivo e o processo metastático (YAQUB & AANDAHL, 2009). O entendimento dessa interação é fundamental para a descoberta de marcadores prognósticos e para o desenvolvimento de novas estratégias terapêuticas (FRIDMAN *et al.*, 2011).

Uma das moléculas presentes no microambiente tumoral e que possuem um papel importante na comunicação de seus componentes são as citocinas. Elas são responsáveis por mediar diversos processos, dentre eles a ativação do sistema imune, o crescimento tumoral e a progressão do neoplasma (MURPHY, 2001), possuindo um papel controverso, o que ressalta a importância de estudos adicionais para determinar seu papel nos diversos tipos de tumores existentes, incluindo os tumores pediátricos.

1.2 QUIMIOCINAS

As quimiocinas são proteínas solúveis e de baixo peso molecular (ROT & VON ANDRIAN, 2004) que constituem uma grande família de mediadores inflamatórios e imunológicos, podendo ser produzidas por leucócitos e células teciduais de forma constitutiva ou induzida (BAGGIOLINI, DEWALD & MOSER, 1997). Estas moléculas se ligam aos seus receptores acoplados à proteína G (GPCRs) a fim de promoverem uma resposta celular, que geralmente culmina com a quimiotaxia (ROT & VON ANDRIAN, 2004). Esse direcionamento leva à movimentação celular durante o desenvolvimento do indivíduo (KLEIN *et al.*, 2001), na homeostasia (VIOLA & LUSTER, 2008) e na inflamação (BALKWILL, 2004).

As quimiocinas são classificadas em quatro famílias de acordo com o arranjo de dois resíduos conservados de cisteína próximo à região N-terminal: CXC, CC, C e CX3C. Estas proteínas são alvos de vários estudos, devido ao seu papel no desenvolvimento e progressão

de doenças como o câncer, particularmente regulando o processo de metástase (ZLOTNIK & YOSHIE, 2000).

1.2.1 A QUIMIOCINA CXCL12 E SEU RECEPTOR CXCR4

O CXCL12 (*Chemokine (C-X-C motif) ligand 12)* é caracterizado por ser uma quimiocina pertencente à família CXC (α-quimiocina) e foi inicialmente denominado fator derivado de células do estroma da medula óssea (SDF-1) e também como um fator pré-estimulador de células B (NAGASAWA, KIKUTANI & KISHIMOTO, 1994). Esta quimiocina, juntamente com o seu receptor CXCR4 (*Chemokine (C-X-C motif) receptor 4)*, atuam em vários processos importantes como a homeostase, desenvolvimento embrionário (TACHIBANA *et al.*, 1998; DOITSIDOU *et al.*, 2002; LU, GROVE & MILLER, 2002) e processos patológicos (MULLER *et al.*, 2001; DEWAN *et al.*, 2006).

Estudos têm demonstrado que esta quimiocina é indispensável para a morfogênese de vários órgãos (NAGASAWA, TACHIBANA & KISHIMOTO, 1998; ANSEL & CYSTER, 2001; SALCEDO & OPPENHEIM, 2003; STUMM & HOLLT, 2007), incluindo o cerebelo (STUMM *et al.*, 2002) e o rim. A expressão do CXCL12 e do seu receptor CXCR4 foi verificada no mesênquima condensado renal, no entanto, após a transição mesenquimal para epitelial a sua expressão diminuiu (TAKABATAKE *et al.*, 2009).

O eixo CXCL12/CXCR4 tem sido muito estudado devido ao seu envolvimento no desenvolvimento tumoral. O CXCL12 possui participação importante no crescimento tumoral, bem como na angiogênese do tumor (ORIMO *et al.*, 2005) e no processo de metástase (MULLER *et al.*, 2001).

O gene *CXCL12* está localizado no cromossomo 10 e contém 4 éxons. Existem pelo menos dois transcritos originados do *splicing* alternativo deste gene: o CXCL12-1a e o CXCL12-1b (SHIROZU *et al.*, 1995). O estudo detalhado do *CXCL12-1b* revelou o polimorfismo no segmento evolucionário conservado da região 3’ não traduzida (3’UTR), (posição 801 G → A) que foi designado como CXCL12 rs1801157. A importância desta alteração reside no fato de a presença do alelo variante aparentemente possuir funções regulatórias importantes, resultantes do aumento na concentração da proteína (WINKLER *et al.*, 1998). Sendo assim, diversos estudos têm avaliado a frequência genotípica do alelo CXCL12-3’A em diferentes populações com o objetivo de avaliar suas possíveis implicações na patogênese tumoral (DE OLIVEIRA *et al.*, 2007; DE OLIVEIRA *et al.*, 2009).

O gene que codifica o CXCR4 está localizado no cromossomo 2q2 e é expresso em diversas células do sistema imune, como as células dendríticas, células T *naive*, células *natural killers* e monócitos, e ainda é o receptor mais comumente expresso nos tumores (LEE *et al.*, 2011). Este receptor também é expresso em células progenitoras durante o desenvolvimento do embrião, permitindo que estas células migrem até o seu local adequado, onde irá se diferenciar em órgãos e tecidos (NAGASAWA *et al.*, 1996).

1.3 FATOR DE TRANSCRIÇÃO FOXP3

A proteína FOXP3 (*Forkhead box protein 3*) é membro de uma família de fatores de transcrição *forkhead/winged-helix*, que possui um papel importante na regulação e desenvolvimento do sistema imune (HORI, NOMURA & SAKAGUCHI, 2003; COFFER & BURGERING, 2004). Esta proteína foi inicialmente descrita como sendo de expressão restrita da linhagem hematopoiética linfoide, principalmente das células T reguladoras (Tregs) (FONTENOT *et al.*, 2005), e até hoje ela é muito utilizada para o isolamento e caracterização desse tipo celular junto com outros marcadores.

As Tregs são consideradas um importante subtipo de células regulatórias, as quais representam acima de 5% dos linfócitos T CD4 do sangue (HOLMES *et al.*, 2008) e estão envolvidas em inúmeros processos, inclusive no câncer (FONTENOT, GAVIN & RUDENSKY, 2003). Uma grande quantidade de células Tregs FOXP3 positivas têm sido encontradas em infiltrados tumorais e no sangue periférico de pacientes com câncer (WOO *et al.*, 2001; LIYANAGE *et al.*, 2002), e estudos sugerem que o envolvimento dessas células pode estar associado a um prognóstico ruim, uma vez que as Tregs estão envolvidas em mecanismos de evasão da resposta imune promovidos pelas células neoplásicas (BATES *et al.*, 2006; CURIEL, 2007; BOHLING & ALLISON, 2008; OHARA *et al.*, 2009).

Embora inicialmente se pensasse que essa proteína era expressa apenas em linhagens linfoides, inúmeros estudos têm demonstrado que a proteína FOXP3 é também expressa em diversos tecidos normais e tumorais (ZUO *et al.*, 2007; EBERT *et al.*, 2008; KARANIKAS *et al.*, 2008), e que a sua localização, nuclear ou citoplasmática, está relacionada ao prognóstico do paciente (TAKENAKA *et al.*, 2013; LOPES *et al.*, 2014).

Diversas doenças têm sido associadas à disfunção do FOXP3, como, por exemplo, doenças autoimunes como a síndrome ligada ao X caracterizada por imunodesregulação, poliendocrinopatia e enteropatia (IPEX) (VAN DER VLIET & NIEUWENHUIS, 2007), o

diabetes melitus 1 (BASSUNY *et al.*, 2003) e doenças autoimunes na tireoide (BAN *et al.*, 2007).

O estudo do gene que codifica para o FOXP3 revelou polimorfismos presentes na região regulatória que podem modificar quantitativamente o produto gênico, podendo levar a disfunção nas Tregs. Os polimorfismos de base única (SNPs) presentes na região intrônica têm sido associados a algumas doenças, como por exemplo a psoríase (GAO *et al.*, 2010) e câncer de mama (LOPES *et al.*, 2014) para o polimorfismo rs 3761548 (-3279 C/A) e o vitiligo (SONG *et al.*, 2013) para o rs2232365 (-924 A/G). Desta forma, a análise das variantes alélicas pode resultar em importantes informações sobre a regulação de transcrição do FOXP3 em diversas patologias, incluindo o câncer.

O grande desafio da oncologia tem sido o entendimento dos mecanismos moleculares que envolvem o desenvolvimento dos tumores malignos. Dentro deste contexto, o estudo de genes como o *FOXP3* e o *CXCL12*, bem como a sua expressão proteica FOXP3 e do seu receptor CXCR4, respectivamente, podem auxiliar na compreensão dos mecanismos moleculares envolvidos no desenvolvimento do TW e do MB. Todos estes parâmetros podem ter relevância clínica e também constituírem alvos promissores na avaliação do prognóstico e no delineamento terapêutico.

2 OBJETIVO

2.1 OBJETIVO GERAL

Este projeto teve como objetivo principal avaliar a presença dos polimorfismos genéticos rs2232365 e rs3761548 e a expressão proteica do fator de transcrição FOXP3, bem como o polimorfismo genético rs1801157 do gene que codifica a quimiocina CXCL12 e a expressão proteica do seu receptor CXCR4 em pacientes pediátricos diagnosticados com meduloblastoma e tumor de Wilms.

2.2 OBJETIVOS ESPECÍFICOS

- Estimar os dados epidemiológicos acerca dos tumores pediátricos do tipo TW, MB, neuroblastoma e tumor de adrenocortical, a partir de um estudo retrospectivo de janeiro de 1990 a dezembro de 2013, na região Norte do Paraná;
- Analisar a associação dos polimorfismos genéticos rs2232365 e rs3761548 do *FOXP3* e rs1801157 do *CXCL12* e a susceptibilidade ao desenvolvimento do TW;
- Analisar a associação entre o polimorfismo rs3761548 do *FOXP3* e o risco de desenvolvimento de MB
- Avaliar a expressão proteica do fator de transcrição FOXP3 e do receptor de quimiocina CXCR4 nos tecidos tumoral e saudável adjacente nos pacientes com TW e MB, através da técnica de imunohistoquímica;
- Avaliar a influência dos polimorfismos genéticos rs2232365 e rs3761548 do *FOXP3* sobre sua expressão proteica, bem como a influência do polimorfismo genético rs1801157 do *CXCL12* na expressão proteica do seu receptor CXCR4;
- Comparar as frequências gênicas e alélicas dos polimorfismos propostos com os dados patológicos dos pacientes (idade, tamanho do tumor e gênero) em ambos os modelos tumorais analisados.

3 MATERIAIS E MÉTODOS

3.1 ASPECTOS ÉTICOS

Este projeto foi aprovado pelo Comitê de Ética em Pesquisa Envolvendo Seres Humanos da Universidade Estadual de Londrina Paraná, Brasil (Nº.171231134.0000.5231). Os indivíduos controles assinaram um termo de consentimento livre e esclarecido para a participação no estudo. Com relação às amostras de pacientes utilizadas no estudo, foi concedida uma autorização por escrito do chefe do Departamento de Patologia Clínica, Hospital Universitário da Universidade Estadual de Londrina, para o uso dos blocos de parafina neste estudo.

3.2 SELEÇÃO DE AMOSTRAS

Foi realizado um estudo retrospectivo de pacientes diagnosticados com MB e TW no Hospital Universitário da Universidade Estadual de Londrina, de Janeiro de 1990 à Dezembro de 2013. Foram selecionadas 41 amostras incluídas em parafinas, sendo 32 de pacientes com TW e 9 amostras de pacientes com MB. Foram realizados cortes dos blocos de tecido, sendo que uma parte foi destinada a extração de DNA e outra à confecção de lâminas para a realização do ensaio de imunohistoquímica.

Para o estudo caso controle, foram selecionados 78 indivíduos livres de neoplasia como controle. Desses indivíduos foi obtido apenas sangue periférico para realizar as análises dos polimorfismos genéticos. Os indivíduos controle são predominantemente Caucasianos, devido ao fato da população prevalente no Sul do Brasil ser proveniente de colonização europeia.

As amostras incluídas em parafina foram selecionadas no Setor de Patologia do Hospital Universitário da Universidade Estadual de Londrina e as amostras controle foram selecionadas no Hospital Universitário da Universidade Estadual de Londrina. Todas as análises foram realizadas no Laboratório de Estudos e Aplicações de Polimorfismos de DNA (LEAP-DNA) e no Laboratório de Patologia Clínica, Hospital Universitário da Universidade Estadual de Londrina.

3.3 EXTRAÇÃO DE DNA DE TECIDO FIXADO EM FORMALINA TAMPONADA E INCLUÍDOS EM PARAFINA

As amostras fixadas em formalina e incluídas em parafina foram extraídas utilizando-se o innuPREP DNA Mini Kit (Analytik Jena AG, Jena, Alemanha), segundo recomendações do fabricante. As amostras de DNA obtidas foram quantificadas por espectrofotometria a 260 nm em espectrofotômetro NanoDrop 2000® (NanoDrop Technologies, Wilmington, DE, EUA). O grau de pureza em relação à contaminação por proteínas foi avaliado pela razão entre as absorbâncias nos comprimentos de 260 nm e 280 nm.

3.4 EXTRAÇÃO DE DNA DE SANGUE PERIFÉRICO

O DNA genômico do grupo controle foi obtido a partir das células do sangue periférico. As amostras foram extraídas utilizando-se o Kit de Extração Mini Spin DNA Genômico (Biometrix, Curitiba, PR, Brasil), segundo recomendações do fabricante. As amostras de DNA obtidas foram quantificadas por espectrofotometria a 260 nm em espectrofotômetro NanoDrop 2000®. O grau de pureza em relação à contaminação por proteínas foi avaliado pela razão entre as absorbâncias nos comprimentos de 260 nm e 280 nm.

3.5 REAÇÃO EM CADEIA DA POLIMERASE (PCR)

Aproximadamente 100 ng de DNA foram amplificados com *primers* específicos para os polimorfismos propostos, sintetizados de acordo com as sequências depositadas no *GenBank*. As reações de amplificação foram realizadas utilizando 100 µM de dNTP, 150 pM de cada iniciador, 1,50 mM de $MgCl_2$, 1,25U de *Taq* DNA polimerase (Invitrogen, Carlsbad, CA, EUA). Todas as reações foram efetuadas com um controle positivo e um controle negativo (ausência de DNA) a fim de assegurar a não contaminação dos produtos de PCR. O termociclador utilizado foi A200 Gradient Thermal Cycler (LongGene, Hangzhou, China).

3.5.1 PCR PARA O POLIMORFISMO RS1801157 DO CXCL12

O polimorfismo rs1801157 também é conhecido como G801A, por ser caracterizado por uma troca de uma guanina por uma adenina na posição 801 (indicado em verde na

sequência abaixo) da região 3'UTR do gene *CXCL12*. Para o estudo deste polimorfismo foram utilizados os seguintes *primers*.

Forward: 5'-CAGTCAACCTGGGCAAAGCC-3'
Reverse: 5'-CCTGAGAGTCCTTTTGCGGG-3'

CAGTCAACCTGGGCAAAGCCTAGTGAAGGCTTCTCTCTGTGGGATGGGATGGTGGAGGGCCACATGGGAGGCTCA
CCCCCTTCTCCATCCACATGGGAGCCGGGTCTGCCTCTTCTGGGAGGGCAGCAGGGCTACCCTGAGCTGAGGCAG
CAGTGTGAGGCCAGGGCAGAGTGAGACCCAGCCCTCATCCCGAGCACCTCCACATCCTCCACGTTCTGCTCATCA
TTCTCTGTCTCATCCATCATCATGTGTGTCCACGACTGTCTCCATGGCCCCGCAAAAGGACTCTCAGG

Os tempos de reação foram os seguintes:

T ºC	Tempo	Ciclos
	Estágio 1	
94ºC	5'	1 ciclo
	Estágio 2	
94ºC	1'	40 ciclos
60ºC	1'	
72ºC	1'	
	Estágio 3	
72°C	10'	1 ciclo

3.5.2 DIGESTÃO ENZIMÁTICA

Após a confirmação da amplificação do fragmento de interesse, foi realizada a digestão enzimática do fragmento utilizando 10 U da endonuclease de restrição *MspI* (Promega Corporation, Madison, EUA) e 10 µL de produto de PCR. Após o preparo da reação, os tubos foram incubados por 4 horas a 37°C. O resultado da digestão foi observado pela clivagem ou não do fragmento de 293 pb.

O sítio de clivagem da enzima *MspI* encontra-se na seguinte sequência:

5'...C▼CGG...3'
3'...GGC▲C...5'

No caso do genoma possuir a troca do nucleotídeo G pelo A, a enzima não reconhecerá o seu sítio de ligação, portanto não haverá clivagem. Sendo assim, o indivíduo homozigoto selvagem sofre clivagem do fragmento de 293 pb em 2 fragmentos: um contendo 193 pb e outro com 100 pb. Já o indivíduo heterozigoto, devido a perda do sítio de clivagem da enzima em apenas um alelo, apresenta 3 bandas com: 293 pb, 193 pb e 100 pb. O

indivíduo que possui o polimorfismo em homozigose, por sua vez, irá apresentar apenas uma única banda de 293 pb.

3.5.1 PCR ALELO ESPECÍFICO PARA POLIMORFISMOS DO GENE FOXP3

Foram analisados dois polimorfismos do gene *FOXP3*: o rs3761548, que é caracterizado por uma troca de uma citosina por uma adenina (C/A) e o rs2232365, caracterizado por uma troca de uma adenina por uma guanina (A/G).

Para o estudo deste polimorfismo foram utilizados os seguintes *primers*.

Polimorfismo -3279 (C/A, rs3761548)

primer sense 5’-CTGGCTCTCTCCCCAACTGA-3’

primer antisense 5’-ACAGAGCCCATCATCAGACTCTCTA-3’*

primer sense 5’-TGGCTCTCTCCCCAACTGC-3’)

primer antisense 5’-ACAGAGCCCATCATCAGACTCTCTA-3’*

* mesmo primer.

CTGGCTCTCTCCCCAACTGAAGGCCTCAGTTTACCCCTCAGCACCCAGAAGGGGGAAGGGGAACCTGGGCTACCA
TTCCCCCTTCTGCCTTCTCACACGTTGGACCCCAACTTCCCACAGGTTGGACGATCCACGATCACAGTGTGGGGC
CCAGCCTCACAAGAGCTGGGCTAGGTGAGGCCCCGGACTCCATAGGTCAGGAGGCCTAGTTGGCCAGAGCGTGGT
GATGATGGAGGCATGTCAGTCAGTCAGGCTGTGTGTCCCCAGAGCTGGTGCTGGTCCCCGAAAACCTTGATTGTG
GGGCCCCTC**TAGAGAGTCTGATGATGGGCTCTGT**

Polimorfismo -924 (A/G, rs2232365)

primer sense 5’-CCCAGCTCAAGAGACCCCA-3’

primer antisense 5’-GGGCTAGTGAGGAGGCTATTGTAAC-3’

primer sense 5’-CCAGCTCAAGAGACCCCG-3’

primer antisense 5’-GCTATTGTAACAGTCCTGGCAAGTG-3’

CCCCAGCTCAAGAGACCCCATCTCTCCTCCTCTCTGTCACTTGCCATGCTGGATCCGTGCATGATCACACTCCTG
GACTCGCCTCCTTGCCCTGAGATCCAGACCCCCGTATTCAGCTGCCCCCTCAGCTCCTCCACTCACATATTTAAT
GCCAGACTCTTCATGTCTATCTACACCTGCACTTTTGCACCCAATCCAACTCCCCGCCATGTCCCCCATCTCAGG
TAATGTCAGCTCGGTCCTTCCAGCTGCTCAAGCTAAAACCCATGTCACTTTGACTCTCCCTCTTGCCCACTACAT

CCAAGCTGCTAGCACTGCTCCTGATCCAGCTTCAGATTAAGTCTCAGAATCTACCCACTTCTCGCCTTCTCCACT
GCCACCAGCCCATTCTGTGCCAGCATCATCACTTGCCAGGACT**GTTACAATAGCCTCCTCACTAGCCC**

As reações de amplificação para ambos os polimorfismos seguiram os seguintes tempos de reação:

T ºC	Tempo	Ciclos
Estágio 1		
94ºC	10’	1 ciclo
Estágio 2		
94ºC	45”	
67ºC	1’	40 ciclos
72ºC	1’	
Estágio 3		
72ºC	10’	1 ciclo

Para o polimorfismo rs3761548, o alelo C amplifica um fragmento contendo 333 pb e o alelo A um fragmento de 334 pb. O polimorfismo rs2232365 amplifica um fragmento de 442 pb para o alelo A e um de 427 pb para o alelo G.

3.5.2 ELETROFORESE EM GEL DE POLIACRILAMIDA 10%

Os produtos da reação tanto alelo específica quanto RFLP foram submetidos à técnica de eletroforese em gel de poliacrilamida 10%, utilizando-se o tampão de carregamento TAE (Tris-Acetato-EDTA) numa voltagem de aproximadamente 60 V por cerca de 2 horas, utilizando 3µL de XC (xileno cianol), 10 µL de cada amostra e utilizando-se sempre um marcador de peso molecular (Ladder – Invitrogen).

3.6 IMUNOHISTOQUÍMICA

Para a análise de imunohistoquímica, as amostras de tecido fixadas em formalina e embebidas em parafina foram cortadas com a espessura de 3 μm, sendo utilizados dois cortes consecutivos que foram fixados em lâmina silanizada (Starfrost, Knittel, Alemanha). As amostras foram aquecidas a 56°C, desparafinizadas em xilol e reidratadas em álcool. A recuperação antigênica foi realizada utilizando tampão de ácido cítrico (1,8mM) e citrato de sódio di-hidratado (8,2mM) e então foram adicionados os anticorpos. O anticorpo utilizado para a marcação do FOXP3 foi um anticorpo monoclonal de camundongo anti-FOXP3 humano (clone 236A/E7; eBioscience, San Diego, CA, EUA), em uma diluição de 1:25, e o

anticorpo para detecção de CXCR4 foi um anticorpo de camundongo anti-CXCR4 (CD184) humano purificado (clone polyclonal; eBioscience, San Diego, CA, EUA), em uma diluição de 1:100. O anticorpo secundário anti-camundongo foi então adicionado, as lâminas foram contra coradas com hematoxilina e a lamínula foi fixada utilizando o balsamo do Canadá. Foram utilizados controles para verificar a especificidade dos anticorpos primários e todas as análises foram feitas por médicos patologistas experientes, que não tinha conhecimento dos dados clinicopatológicos do paciente. As amostras foram classificadas em positivas ou não para a marcação.

3.7 ANÁLISE ESTATÍSTICA

O estudo de associação de suscetibilidade ao desenvolvimento do TW foi calculado através da análise de Odds ratio (OR), adotando um intervalo de confiança (IC) de 95%, e também através do teste exato de Fischer, utilizando o programa estatístico Prism 6.0 for Windows, versão 6.01 (GraphPad Software, San Diego, CA, EUA). Para a análise da relação entre os resultados obtidos com a imunohistoquímica, os polimorfismos genéticos e as características patológicas, foi utilizado o teste estatístico Chi quadrado utilizando o programa SPSS Statistics 20.0 software (SPSS inc., Chicago, IL, USA). O valor de *p* foi considerado estatisticamente significante quando menor que 0,05.

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5 PRODUÇÃO BIBLIOGRÁFICA

5.1 ARTIGO 1

ABSTRACT

Chemokines and its receptors have significant impact on physiological and pathological processes, and studies concerning their association with tumor biology are subject of great interest in scientific community. CXCL12/CXCR4 axis has been widely studied due to its significant role in tumor microenvironment, but it is also important to development and maintenance of tissues and organs, for example, in the brain and cerebellum. Studies have demonstrated that CXCL12 and CXCR4 are required for normal cerebellar development, and that dysfunction in this pathway may be involved with medulloblastoma pathogenesis. In this context, a new molecular subgroup has been suggested based on the importance of the association between CXCR4 overexpression and sonic hedgehog subgroup.

Treatment using CXCR4 antagonists showed significant results, evidencing the important role and possible therapeutic capacity of CXCR4 in MB. This review summarizes studies on MB cell biology, focusing on a chemokine-receptor axis, CXCL12/CXCR4, that may have implications for treatment strategies once it can improve life expectancy and reduce neurocognitive sequelae of patients with this neoplasia.

INTRODUCTION

Tumors originate from cells with dysregulation on normal growth mechanism control, caused by genetic mutations (DVORAK, 1986). Studies have demonstrated that the tumor microenvironment is constituted not only by tumor cells, but also of extracellular matrix, fibroblasts, endothelial cells, and immune cells that could influence tumor progression (RONNOV-JESSEN *et al.*, 1995; QUAIL & JOYCE, 2013).

One chemokine that has been acquiring relevance in cancer is CXCL12, whose receptor CXCR4 is overexpressed in at least twenty different human cancers, including breast cancer, ovarian cancer, melanoma and prostate cancer (BALKWILL, 2004). Besides their critical role in tumor cell growth (BARBERO *et al.*, 2003), survival and angiogenesis in multiple cancers (BALKWILL, 2003), this chemokine receptor has been described as an important homing and metastatic mediator of secondary growth in organs that produce

CXCL12, such as liver (YU *et al.*, 2006), lung and bone marrow (MULLER *et al.*, 2001). However, the contribution of CXCR4/CXCL12 axis in organ-specific dissemination and tumor growth has been strongly debated (ZEELENBERG, RUULS-VAN STALLE & ROOS, 2003; AIROLDI *et al.*, 2006; ARSCOTT *et al.*, 2009).

Studies have shown that CXCR4, a molecule strongly expressed in proliferating granule neuron precursors (GNP), that are cell types associated with medulloblastoma (MB) (SCHULLER *et al.*, 2005), is also involved with sonic hedgehog (SHH) pathway (ZHAO *et al.*, 2002), as well as its ligand, that significantly enhances SHH–induced cell proliferation (KLEIN *et al.*, 2001; SENGUPTA *et al.*, 2012). CXCR4 is predominantly expressed in tumor areas, while CXCL12 is expressed mainly in the endothelium of tumor associated blood vessels (RUBIN *et al.*, 2003; YANG *et al.*, 2008). It has also been described another receptor for the chemokine CXCL12, the CXCR7, however, it seems to play no role in this tumor (SENGUPTA *et al.*, 2012).

CXCL12 and CXCR4 are expressed in several brain tumors, including MB and the expression level of this receptor appears to have prognostic significance (RUBIN *et al.*, 2003; CALATOZZOLO *et al.*, 2006; BIAN *et al.*, 2007). Positive CXCR4 expression was identified in nine of ten samples of MB tumors, in contrast to little or no staining in normal cerebellum counterparts (RUBIN *et al.*, 2003). Studies also demonstrated that fetal cerebellum has a high expression of CXCR4, which decreased significantly with age (SCHULLER *et al.*, 2005; SENGUPTA *et al.*, 2012). Therefore, this review summarizes studies reporting the importance of CXCL12 and CXCR4 axis on cerebellum development in order to strengthen the relevance of this pathway for MB development.

CXCL12 and CXCR4

Beyond their function in leukocytes trafficking, particularly during infection and inflammation (BALKWILL, 2004; BARBIERI *et al.*, 2008; VIOLA & LUSTER, 2008), chemokines are involved in several processes, including migration, proliferation and survival during development (KLEIN *et al.*, 2001) as well as pathological states (BALKWILL, 2003) and homeostasis (VIOLA & LUSTER, 2008).

The chemokine superfamily comprises soluble small peptides and is structurally classified based on two conserved cysteine residues in the N-terminal region. These residues may be separated by one or three amino acids, adjacent, or harboring only one N-terminal cysteine residue (CXC, CX3C, CC or XC respectively) (ZLOTNIK & YOSHIE, 2000).

Regarding their function, chemokines can also be classified into inflammatory or homeostatic chemokines, and their role in tumor biology has been the subject of numerous studies worldwide (KRYCZEK *et al.*, 2007; FISCHER *et al.*, 2009).

Chemokines act by binding to the respective G-protein-coupled seven-span transmembrane receptors (GPCRs), through activation of classical MAP-kinase (PETRAI *et al.*, 2008) or PI3-kinase/PKC signaling cascades (TEICHER & FRICKER, 2010). This signaling results in transcription of target genes involved in cell invasion (BARTOLOME *et al.*, 2004), motility (REILAND, FURCHT & MCCARTHY, 1999), interactions with extracellular matrix (IVANOFF, TALME & SUNDQVIST, 2005) and survival (KRATHWOHL & KAISER, 2004).

In addition to their role in the immune system, chemokines are also implicated in the development and maintenance of a variety of tissues and organs, including the central nervous system (CNS), influencing migration and differentiation of several cell types (REISS *et al.*, 2002; HATTERMANN *et al.*, 2008). Chemokines receptors and ligands such as CCL2/MCP-1, CCL5/RANTES and CXCL12/CXCR4 have been described to be expressed in several parts of the brain (ANDJELKOVIC & PACHTER, 2000; VAN DER MEER *et al.*, 2000) wherein the latter was also present in the cerebellum (STUMM *et al.*, 2002). Furthermore, there are supporting evidences showing that chemokines may influence the crosstalk between neuron and glial cell types, under physiological and pathological conditions and there are also suggestions that they can function as a third communication system in the brain (in addition to neurotransmitters and neuropeptides) (ADLER & ROGERS, 2005; ROSTENE *et al.*, 2011).

CXCL12 is a CXC chemokine that was first cloned from a bone marrow-derived stromal cell line (firstly called SDF-1) and identified as a pre-B cell growth stimulating factor. CXCL12 is widely expressed in a variety of tissue types acting as a potent chemoattractant at different stages of development (SECCHIERO *et al.*, 2000; WRIGHT *et al.*, 2005). Four human isoforms of CXCL12, with specific expression in some tissues, have been reported. They are derived from alternative splicing events and share the same first three exons, described as CXCL12γ (gamma), CXCL12δ (delta), CXCL12ε (epsilon), and CXCL12φ (phi) (YU *et al.*, 2006).

CXCR4 is a highly conserved GPCR that binds to the ligand CXCL12 (OBERLIN *et al.*, 1996). CXCR4 has received considerable attention since it acts as a co-receptor for entry of HIV viruses that target $CD4^+$ T cells (FENG *et al.*, 1996). During development, CXCR4 is expressed in a broad range of tissues, including immune and central nervous systems, and the

importance of this axis during development has been evidenced once its deficiency leads to circulatory, hematopoietic, immune, and CNS defects (DOITSIDOU *et al.*, 2002).

CXCL12 and CXCR4 in normal cerebellum development

The cerebellum is a CNS structure whose development continues to occur in postnatal period, leading itself vulnerable to malformation events. The external granule cell layer (EGL) is formed during cerebellar development, when cerebellar granule cell progenitors, produced in the Upper Rhombic Lip (URL), migrate over the cerebellar primordium to form a secondary proliferative zone, the EGL. The formation of the internal granule cell layer (IGL) occurs during early postnatal development, when granule cell precursors in the outer zone of EGL proliferate, migrate to the inner zone of EGL and exit from cell cycle, differentiate, and radially migrate through the Purkinje cell layer to their final destination (HATTEN & HEINTZ, 1995; WINGATE, 2001).

The role of CXCL12 in other parts of the nervous system includes acting as a strong chemoattractant for granule cell progenitors in the URL, EGL, and dentate gyrus (BAGRI *et al.*, 2002; ZHU *et al.*, 2002). This chemokine also plays a critical role during neurogenesis through promotion of axonal growth (TAKEUCHI *et al.*, 2007; OHSHIMA *et al.*, 2008) and attraction of neuroblasts from the subventricular zone into an injured site (FILIPPO *et al.*, 2013).

The chemokine CXCL12 is produced *in vivo* by the meningeal cells, near the URL and EGL cells, being expressed in embryonic and postnatal meninges covering the cerebellum (REISS *et al.*, 2002; ZHU *et al.*, 2002). It is known as a strong chemoattractant to URL cells, preventing the migration of CXCR4-expressing premature granule cells away from the EGL (ZOU *et al.*, 1998; KLEIN *et al.*, 2001). The irregular EGL formation could partially be attributed to defects on cell migration from URL to EGL (ZHU, YU & RAO, 2004).

Studies have shown that CXCR4 protein is expressed in EGL proliferating precursors in fetus, but not in granule cells of adult cerebellum (SCHULLER *et al.*, 2005). Experiments with CXCR4 and CXCL12 deficient mice showed abnormal cerebellar development, with malformations including irregular EGL, ectopic Purkinje cells, and abnormally migrated granule cells (ZOU *et al.*, 1998) displaying, thus the importance of this chemokine receptor in cerebellar development.

CXCL12 also cooperates with SHH, a potent mitogen produced by Purkinje cells, to stimulate the proliferation of granule cells (KLEIN *et al.*, 2001). Therefore, the involvement

of CXCL12/CXCR4 axis with SHH pathway appears to be important in normal and also in tumor development of the cerebellum (SENGUPTA *et al.*, 2012).

CXCL12 and CXCR4 involvement in medulloblastoma

Medulloblastoma is considered the most frequent brain tumor in childhood, being recognized as major cause of morbidity in children (RICKERT & PAULUS, 2001; DHALL, 2009). It can be classified according to its histology as classic, desmoplastic, anaplastic and extremely nodular, and also according to its gene expression profile, as molecular subgroups named WNT, SHH, Group 3 and Group 4, which differ in several aspects, such as demographics, histology, DNA copy number aberrations, and clinical outcome (KOOL *et al.*, 2012). The identification of molecular subgroups is important concerning the clinical management, which includes patient stratification, treatment strategy, and design and implementation of targeted therapy (LI, LAU & NG, 2013).

Classic medulloblastoma (CMB) and desmoplastic medulloblastoma (DMB) tumors account for the vast majority of cases, and MBs with extensive nodularity (MBEN) and large cell MBs are considered rare (GIANGASPARO *et al.*, 2000). It is known that different histologies can be associated with distinct outcomes, once DMB tumors possess superior survival and anaplastic large cell tumors possess inferior survival, compared with CMB tumors (HATTEN & ROUSSEL, 2011).

Some histologically classified subtypes of MBs are particularly associated with specific transcription profile, as DMB tumors are correlated with dysfunctions in genes of SHH-Patched pathway (WECHSLER-REYA & SCOTT, 2001) and large cell and anaplastic variants are associated with overexpression of c-Myc (HERMS *et al.*, 2000; EBERHART *et al.*, 2004).

When it was investigated the relation between CXCR4 expression and molecular subtypes, Sengupta and coworkers found that this chemokine receptor was present at WNT and SHH subtype tumors, but only the SHH subtype tumors displayed relative CXCR4 overexpression, suggesting that CXCR4 signaling depends on SHH for its activation in MB (SENGUPTA *et al.*, 2012). It probably occurs due to the involvement of SHH in CXCR4 signaling pathway, more specifically interfering in CXCR4 surface expression through proteins responsible for its desensitization control, like GRK2 or GRK6 (YUAN *et al.*, 2013), both in normal and tumor cerebellar cells.

Sengupta and coworkers even proposed a new molecular subgroup classification, considering the SHH-CXCR4 high expression tumors, once it correlated with prognostically important features like histology and age (SENGUPTA *et al.*, 2012). The predominance of CXCR4 high expression in infants is nearly half, compared to only 13% of low expression group in this age. It was also observed that certain tumor histology subtype correlates with high CXCR4 gene and protein expression in MB, like DMBs and MBENs (SCHULLER *et al.*, 2005; SENGUPTA *et al.*, 2012). This makes sense since DMBs tumors derived from cerebellar EGL, which are cellular types that require CXCR4 and CXCL12 expression for its development.

Different reports about the role of CXCR4 in patient's outcomes have been published. One studied showed that SHH MB with desmoplastic histology has a favorable prognosis, whereas non-desmoplastic SHH MB has higher rates of metastasis and an intermediate prognosis (KOOL *et al.*, 2012). Although, it was also reported that CXCR4 is expressed in the c3/SHH MB molecular subgroup and also that this subgroup together with the c1 are related to the majority of relapses and death due to MB progression (CHO *et al.*, 2011).

Yuan and coworkers showed the involvement of PDGFR in the activation of CXCL12-CXCR4 signaling in SHH responsive MB cells and also the possible role of GF receptor/PDGFR-Src mediating suppression of GRK6, which will promote CXCR4 signaling and cell migration (YUAN *et al.*, 2013). Thus, the capacity of CXCL12 and CXCR4 to regulate proliferation and migration of neural precursor cells raises the possibility of these molecules be considered as therapeutic targets in malignancies arising from CNS progenitors (RUBIN *et al.*, 2003).

Studies have verified that AMD 3100, a small-molecule that possess the ability to inhibit CXCR4 activation, has a significant anticancer activity, since it reduces activation of extracellular signal-regulated kinases 1 and 2 (Erk 1/2) and Akt, and also increased rates of apoptosis in MB, *in vivo*.(RUBIN *et al.*, 2003) Yang and coworkers also showed that AMD 3465, a competitive antagonist of CXCR4, forskolin (an adenylyl cyclase activator) and PDE4 (cAMP inhibitor) have the capacity to block *in vivo* and *in vitro* tumor growth, evidencing the important and possible role of CXCR4 in MB treatment (YANG *et al.*, 2008).

CONCLUSION

The chemokine CXCL12 and its receptor CXCR4 play an important role in cerebellar development, both in physiological and pathological conditions and their association with SHH pathway may be enlightening, concerning the development of MB belonging to SHH molecular subgroup. The use of CXCR4 inhibitors as a promising anticancer therapy have been suggested in recent years, and the results seem to have significant impact on MB growth, confirming the importance of CXCL12/CXCR4 axis in this tumor.

Along the way, chemokine and its receptors have been discovered in neural and other cells of the CNS. With the recognition that chemokines and their receptors on these cell types could interact during pathological processes, the cross-system signaling between the CNS and the immune system becomes apparent. Deciphering this signaling will assist researchers to deploy their insights in service of novel strategies to treat neurological disease.

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5.2 ARTIGO 2

Epidemiology of pediatric solid tumors: neuroblastoma, nefroblastoma, medulloblastoma and adrenocortical tumors in North Paraná

ABSTRACT

Pediatric tumors corresponds to 1 to 3% of total cases of cancer, and in Brazil, it accounts for almost 3%. Since pediatric tumors present very specific characteristics and histopathological origins, this group of cancer should be studied apart from those that affect adults. The cure rates for childhood cancers are impressive compared with those for adult malignancies, however, the treatment strategies are usually associated with major side effects and can ultimately reduce the life quality for survivors. Descriptive and epidemiological studies are important and contribute to the characterization of various types of tumors in populations. Therefore, the aim of this study was to evaluate the incidence of neuroblastoma (NB), Wilms' Tumor (WT), medulloblastoma (MB) and adrenocortical tumors (ACT) in the pediatric population of North Paraná, Brazil. The clinic and pathological data of the tumors were obtained from a retrospective study from January 1990 to December 2013. Clinical characteristics analyzed were age at diagnosis, gender and type of tumor. It was found 88 cases of solid pediatric tumors and the frequencies observed for tumors analyzed were: WT (43.182%), NB (39.773%), MB (6.818%) and ACT (10.227%). WT mean age was 3.51 years old and the male/female ratio was 0.73. The mean age for NB cases was 3.48 years old, with almost 74% of them occurring under 4 years old and the male/female ratio being 1.18. MB patients showed a mean age at diagnosed of 6.388, affecting both male and females equally. The mean age for ACT was 8.29 years old, with almost 45% of patients being diagnosed before 4 years old and a male/female ratio of 0.50. This study will help to construct a panel of pediatric tumor incidence, together with some clinical pathological data, which will facilitate inter-regional comparisons that can benefit both pediatric and public health.

Keywords: epidemiology, pediatric tumor, Wilms' tumor, neuroblastoma, medulloblastoma, adrenocortical tumor.

INTRODUCTION

The estimated incidence of pediatric tumors all over the world varies from 1% to 3% total cases of cancer. In Brazil, the median percentage observed is close to 3% and it was estimated, for 2014, approximately 11,840 new cases of cancer in children and adolescents up to 19 years (INCA, 2014). The latest information available for mortality showed 2,740 deaths by cancer (2010) in this group, and it is considered the leading cause of death by disease in children from 1 to 19 years old (INCA, 2011).

Pediatric tumors present very specific characteristics and histopathological origins, thus this group of cancer should be studied separately from those that affect adults. Regarding to their clinical behavior, tumors in children and adolescents generally present short latency periods, rapidly growth, are more aggressive, but respond better to treatment and are considered good prognosis (MALKIN, 1997).

The overall cure rate of childhood cancer now stands at approximately 80%, mainly due to the use of cytotoxic chemotherapy and radiotherapy, but this approach are often associated with major side effects, and can ultimately reduce the quality of life for survivors. Although cure rates for childhood cancers are impressive relative to those for adult malignancies, cancer remains the leading cause of death by disease among children over 1 year of age in developed countries (PUI *et al.*, 2011; HUDSON *et al.*, 2012).

The spectrum of cancers occurring in the pediatric population is markedly different from that seen in adults. For example, the major brain and solid tumors that arise in children, including medulloblastoma (MB), neuroblastoma (NB) and Wilms' tumor (WT), are exceedingly rare in adults (DOWNING *et al.*, 2012). The incidence of adrenocortical tumor (ACT) is estimated at 1 to 2 cases per 1 million/inhabitants a year, nevertheless, a high incidence of adrenocortical tumors affecting children and adults has been observed in the Southern and Southeastern Brazil (10 to 15 times greater than worldwide incidence) (RODRIGUEZ-GALINDO *et al.*, 2005).

Therefore, the aim of this study was to evaluate the incidence of the MB, WT, NB and ACT in the pediatric population of North Paraná, Brazil.

METHODOLOGY

The clinical and pathological data of the childhood tumor (WT, MB, NB and ACT) patients were retrospectively, retrieved and reviewed from the registries and files of the

Department of Pathology, Clinical and Toxicological Analysis at the University Hospital of State University of Londrina during the period from January 1990 to December 2013, following approval from the Institutional Human Research Ethics Committee of the State University of Londrina, Paraná, Brazil (CAAE Nº.171231134.0000.5231). The clinical characteristics analyzed were age at diagnosis, gender and type of tumor.

RESULTS AND DISCUSSION

This retrospective study evaluated eighty-eight pediatric patients with pathologically confirmed solid tumors. Of all tumors analyzed, forty seven (53.409%) were female and forty one (46.591%) were male, with a mean age of 4.515 years (range 1 month - 18 years). Figure 1A shows the frequencies observed for NB, WT, MB and ACT.

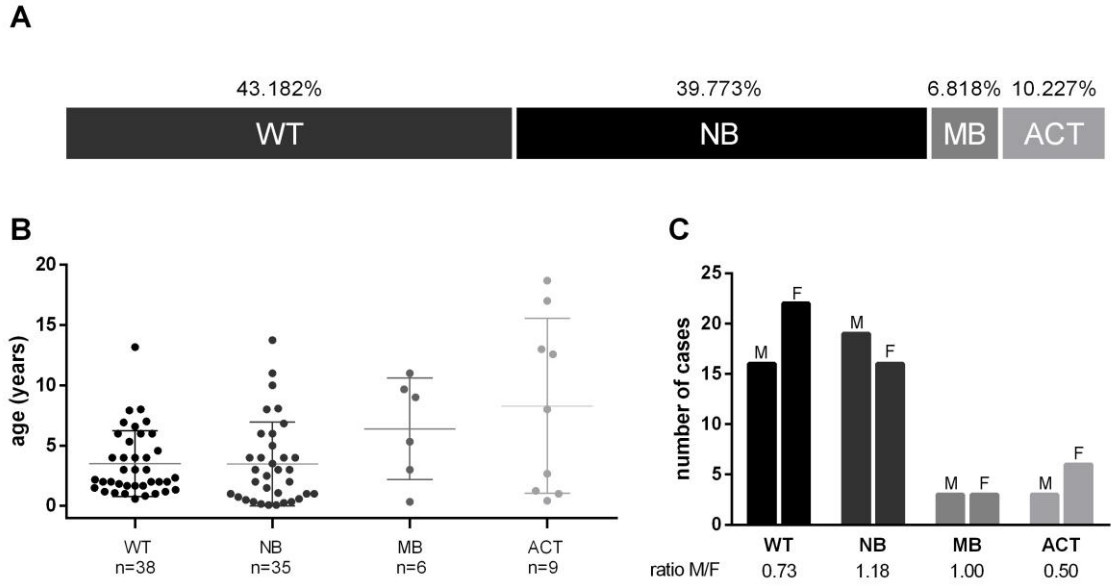

Figure 1: Childhood solid tumor distribution in North Paraná. A: distribution of the 88 pediatric solid tumors found in North Paraná between january 1990 and december 2013. **B**: Age scatter dot plot for the four solid tumors analyzed. Bars represents the mean age with standard deviation. **C**: number of Wilms' tumor, neuroblastoma, medulloblastoma and adrenocortical tumor cases found, divided by gender and the ratio between male and female occurrence (M/F). WT: Wilms' tumor, NB: neuroblastoma; MB: medulloblastoma, ACT: adrenocortical tumor, M: male, F: female.

The WT, also named as nephroblastoma, is a complex mixed embryonic neoplasm of the kidney and the most common renal tumor of the childhood, accounting for 6% of all childhood cancer, affecting 1 in 10,000 children, especially among those younger than 6 years. The mean age at diagnosis was 3.5 years (more than 80% of cases is diagnosed before

age of 5), with an annual incidence of approximately 7 to 10 cases per million. The overall survival rate is about 90% (BRESLOW *et al.*, 1988).

The most important determinants of outcome in children with WT are tumor stage and histopathology. The majority of patients with WT have favorable histology associated with a good prognosis. A small percentage of patients have anaplastic tumors associated with resistance to chemotherapy and a poor prognosis even when the tumor is confined to the kidney. Concerning the data observed for WT patients, the mean age at tumor presentation was 3.513 years, with more than 76% of the cases diagnosed before the age of 5, which is in accordance with literature data (BRESLOW *et al.*, 2006).

NB is derived from embryonic neural crest cells that form the peripheral sympathetic nervous system and have a high potential to migrate, and is the most common solid extra-cranial neoplasm of childhood, representing 8-10% of all pediatric cancer. Approximately 10.5 per million children younger than 15 years of age are affected (PARK, EGGERT & CARON, 2010). At presentation, 50% of children are under age of 2 years, 75% under age of 4 years, and 90% under age of 10 years. A more favorable prognosis is recognized for children younger than 15-18 months of age than for older children (SHARP, GELFAND & SHULKIN, 2011). Regarding to NB tumor patients observed in this study, the mean age at presentation in our sample was 3.48 years, with 73.529% of the patients under age of 4 years. This data is in accordance with literature data, which indicates that at presentation, 75% of children diagnosed with NB are under age of 4 years (SHARP, GELFAND & SHULKIN, 2011).

MB is one of the most common malignant brain tumors of childhood (MCNEIL *et al.*, 2002) and consist of small, round blue cells resembling immature neural progenitor cells on pathological examination and accounts for approximately 20% of all brain tumors in children and adolescents (OSTROM *et al.*, 2013). In young adults, this tumor accounts for less than 2% of central nervous system. The incidence of MB is about 1000 new cases every year worldwide and a mean age between 3 and 7 years (FOGARTY *et al.*, 2007).

MB is a heterogeneous class of embryonic tumors, which include subgroups with genetic anomalies in developmental pathways that are critical for normal cerebellar development. It can be classified in five histopathologic subgroups, including the classic subgroup with some differentiated neurons, the desmoplastic variant in which tumor cells show some differentiation and are surrounded by extracellular matrix, MBs with nodularity, and the anaplastic and large cell anaplastic forms, the most aggressive of the disease that invariably connotes poor prognosis (NORTHCOTT *et al.*, 2011). In addition, four molecular

subgroups were identified, based on the expression profile (SHH, WNT, group 3 and group 4) and overlap the histopathologic subgroups (KOOL *et al.*, 2008). The data observed for medulloblastoma patients differs from the other types of tumor in this study. Besides the six childhood patients found, which were under age of 12 (mean 6.388), we also found six adult patients diagnosed with medulloblastoma which had 20 years or more at presentation (data not shown).

ACT are rare but aggressive in children and adolescents, accounting for less than 0.2% of all pediatric neoplasms and 1.3% of all carcinomas in patients less than 20 years old (RIBEIRO & FIGUEIREDO, 2004). Approximately 65% of ACT occurs in children younger than 5 years old (WOOTEN & KING, 1993). A high incidence of adrenocortical tumors affecting children and adults has been observed in the Southern and Southeastern Brazil (10 to 15 times greater than global incidence) and is associated with an inherited *TP53* mutation (p.R337H) (RIBEIRO *et al.*, 2000; SANDRINI *et al.*, 2005). Unlike pediatric carcinomas in general, which show a progressive increase in incidence with age, literature has been indicating that ACT has a peak incidence between ages 0 and 5 years (WOOTEN & KING, 1993). However, in our sample we observed a mean age of 8.288 years, with only 44.445% (4/9) of patients diagnosed before 4 years.

CONCLUSION

Descriptive and epidemiological studies are important and contribute to the characterization of various types of tumors in populations. In addition, providing a better understanding of childhood tumor patient profiles in North region of Paraná state together with others similar studies will help to form larger series and facilitate inter-regional comparisons by collecting data from centers that surgically treat pediatric tumors, thereby benefitting both pediatric and public health.

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5.3 ARTIGO 3

Genetic polymorphism and protein expression of transcription factor FOXP3 and protein expression of chemokine receptor CXCR4 in medulloblastoma patients

ABSTRACT

Medulloblastoma (MB) is the most frequent malignant brain tumor in children. There are evidences indicating that the abnormal expression of chemokine receptors such as CXC chemokine receptor-4 (CXCR4) is associated with the development, progression and metastasis of tumor cells. However, the role of CXCR4 in medulloblastoma remains unclear. The transcription factor forkhead box P3 (FOXP3) is expressed in regulatory T cells and also in tumor cells, and its role in tumor behavior is controversial. The identification of single nucleotide polymorphisms (SNPs) in *FOXP3* regulatory region may involve changes in this protein expression. The purpose of this study was to verify MB patient's genotype for the *FOXP3* polymorphism rs3761548 and also verify the FOXP3 and CXCR4 protein expression in these samples. Although MB is considered rare in adults, our samples included six adult and 3 children patients. Two childhood patients presented the rare AA homozygous genotype and one was carrier for this allele, while in adults homozygous CC genotype was prevalent and all patients were allele C carriers. Some samples showed expression for both CXCR4 and FOXP3 in tumor cells, including one adult patient that had relapsed after 8 years at diagnosis. Albeit it was not possible to address the role of these proteins in medulloblastoma, this study showed for the first time the expression of FOXP3 in medulloblastoma tumor cells and also the genotype distribution of *FOXP3* polymorphism rs3761548 among adults and children. Additional studies adopting a bigger sample could give a better understanding about the role of these proteins in medulloblastoma microenvironment.

Keywords: medulloblastoma, CXCR4, FOXP3, genetic polymorphism, protein expression.

INTRODUCTION

Medulloblastoma (MB) is recognized as major cause of morbidity in children and is the most common malignant brain tumor of childhood (among children aged less than 19 years), accounting for approximately 20% of all primary tumors of the central nervous system (BRANDES, PALMISANO & MONFARDINI, 1999). In young adults, this tumor accounts for less than 2% of central nervous system (OSTROM *et al.*, 2013).

The interactions that occur in the tumor microenvironment involves the immune system, the malignant neoplastic cells and the surrounding stroma, and the complex crosstalk between these cells dictates the growth, invasiveness and metastatic process of tumor cells (YAQUB & AANDAHL, 2009). Thereby, the understanding of these complex networks is extremely relevant for the discovery of prognostic markers and the development of new therapeutic strategies (FRIDMAN *et al.*, 2011).

The role of chemokines has been described in inflammatory or homeostatic states and their role in tumor biology has been the subject of numerous studies worldwide (KRYCZEK *et al.*, 2007; FISCHER *et al.*, 2009; DE OLIVEIRA *et al.*, 2013). It was also observed that they play a major role in some organs development, for example, the cerebellum (SCHULLER *et al.*, 2005).

During development, CXCR4 and its ligand CXCL12 are expressed in immune and central nervous systems, and the importance of this axis has been evidenced once its deficiency leads to circulatory, hematopoietic, immune, and central nervous system (CNS) defects (DOITSIDOU *et al.*, 2002). Experiments with CXCR4 and CXCL12 deficient mice showed abnormal cerebellar development, with malformations including irregular EGL (external granule cell layer), ectopic Purkinje cells, and abnormally migrated granule cells (ZOU *et al.*, 1998) highlighting, thus, the importance of this axis in cerebellar development.

The transcription factor FOXP3 has an important role in the modulation of the immune response and in peripheral tolerance, being highly expressed by Treg cells (SAKAGUCHI, 2000). These cells play an important role in the control of cancer immunity through infiltration of tumors along with other immune cells, in order to limit inflammation and evade immunosurveillance (BOS & RUDENSKY, 2012). This protein was also described to be expressed in tumor cells (ZUO *et al.*, 2007; EBERT *et al.*, 2008; KARANIKAS *et al.*, 2008), and its localization (nuclear or cytoplasmic) appears to be related to patients prognosis (TAKENAKA *et al.*, 2013; LOPES *et al.*, 2014).

Mutations in *FOXP3* gene could change FOXP3 functionally or quantitatively (GAO *et al.*, 2010), therefore leading to altered counting of $CD4^+CD25^+$ Tregs or either in its location along the tumor and normal cells (WANG *et al.*, 2009).

Considering the importance of tumor microenvironment permittivity and anti-tumor immune responses in cancer development, we investigated whether *FOXP3* polymorphism rs3761548 is presented in MB and if there are CXCR4 and FOXP3 protein expression in these patients from University Hospital from State University of Londrina localized at Londrina, Paraná, Brazil.

MATERIAL AND METHODS

Human Subjects

Following approval from the Institutional Human Research Ethics Committee of the State University of Londrina, Paraná, Brazil (Nº.171231134.0000.5231), it was obtained clinical-pathological information and paraffin embedded tissues containing tumor and normal adjacent tissue from nine MB patients diagnosed at University Hospital of the State University of Londrina, Paraná State, Brazil, in a retrospective study from January 1990 to December 2013.

DNA Extraction

The genomic DNA was isolated from formalin-fixed paraffin embedded samples according innuPREP DNA Mini Kit (Analytik Jena AG, Jena, Germany) protocol. The DNA was quantified by NanoDrop 2000c® Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) at a wavelength of 260/280 nm.

FOXP3 Genetic Polymorphism rs3761548

Allele specific-polymerase chain reaction (AS-PCR) analysis was performed to determine the genotype of the polymorphism rs3761548 of *FOXP3*, as described previously (GAO *et al.*, 2010), with modifications. Approximately 100 ng of DNA was amplified by polymerase chain reaction (PCR) with specific primers for *FOXP3* (Table 1) following the GenBank accession number NG_007392. The samples were amplified using dNTP 100 µM,

150 pM of each primer, $MgCl_2$ 1,5 mM, buffer 10% and 1.25 units of Taq polymerase (Invitrogen, Carlsbad, CA, USA). PCR conditions were 10 min denaturation at 94°C, followed by 40 cycles of 30s at 94°C, 30s at 67°C and 1min at 72°C, and 10 min elongation at 72°C in a thermocycler A200 Gradient Thermal Cycler (LongGene, Hangzhou, China). This polymorphism consists in a change from cytosine to adenine in the position -3279 of *FOXP3*. The products amplified consist of a 333 base pairs for C allele and 334 base pairs for A allele and were analyzed by electrophoresis on acrylamide gel (10%), finally detected by a silver staining method.

Table 1: Primers sequences and PCR products used in genetic variant analyze of *FOXP3* gene

Polymorphism	Primers sequence	PCR product
rs3761548 (*FOXP3*)	5'-CTGGCTCTCTCCCCAACTGA-3'	Allele A 334bp
	5'-ACAGAGCCCATCATCAGACTCTCTA-3'	
	5'-TGGCTCTCTCCCCAACTGC-3'	Allele C 333bp
	5'-ACAGAGCCCATCATCAGACTCTCTA-3'	

Immunohistochemical Staining

The FOXP3 and CXCR4 status were analyzed immunohistochemically on formalin-fixed, paraffin-embedded tumor sections, using FOXP3 and CXCR4 antibodies at a dilution of 1:25 and 1:100, respectively. For immunohistochemical analysis, 3 μm of tissue sections was obtained from MB tumor samples and mounted on a Silanized Slide (Starfrost, Knittel, Germany). Samples were heated at 56°C, deparaffinized in xylene, and rehydrated in a graded alcohol series. Antigen retrieval was performed with citrate/citric acid buffer and then, a mouse monoclonal antibody anti-human FOXP3 (1:25) (clone 236A/E7; eBioscience, San Diego, CA, USA) and a mouse antibody anti-human (CD184) CXCR4 (1:100) (clone polyclonal; eBioscience, San Diego, CA, USA) were used. The sections were stabilized at room temperature for 30 min and washed with PBS (phosphate buffered saline) and anti-mouse/rabbit HRP secondary antibody was used as second step (Bio SB Inc. Santa Barbara, CA, USA). The diaminobenzidine (DAB) chromogen system was used (Bio SB Inc. Santa Barbara, CA, USA) and counter staining was performed with hematoxylin. The slides were completed setting the coverslips using Canada balsam. Controls were performed to verify the specificity of primary antibody and all analyses were made by experienced pathologists, who were blinded to the clinicopathological data. The staining was established as absent or present.

RESULTS

Regarding gender, our sample included 4 males and 2 females for adult patients and 2 males and 1 female for children patients. The age among adult patients ranged from 20 to 29 (mean age = 23.66) years old while the children was 5 to 11 (mean age = 8.33) years old. Tumor size ranged from 1.5 to 3 cm.

A polymerase chain reaction (PCR) analysis of the *FOXP3* rs3761548 was performed for the DNA samples and the patterns of amplicons are shown in Figure 1.

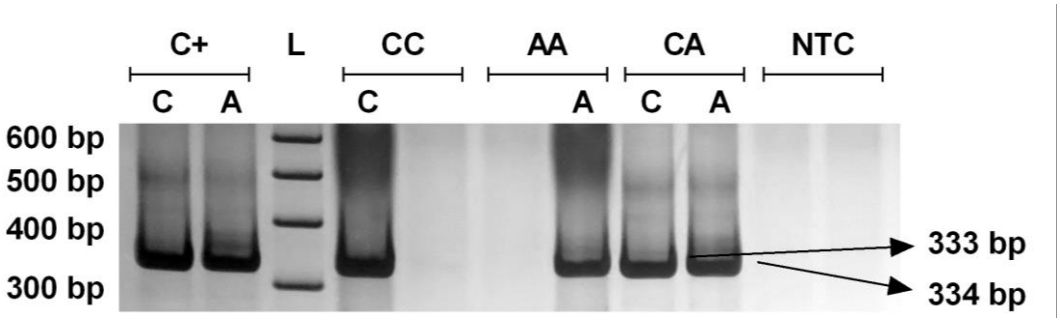

Figure 1. Analysis of *FOXP3* polymorphism. Electrophoretic profile for *FOXP3* rs3761548; C+: positive control; L: Ladder 100bp; CC: Homozygote individual; CA: Heterozygote individual; AA: Homozygote individual NTC: no template control.

Genotype profile of MB samples and immunohistochemical assay for FOXP3 and CXCR4 are shown in Table 2.

Table 2. Analysis of FOXP3 genetic polymorphisms and its protein expression and CXCR4

Samples	*FOXP3* rs3761548 genotype	CXCR4 Expression (Tumor)	FOXP3 Expression (Tumor)
A1	CA	-	+
A2	CC	-	-
A3	CA	-	+
A4*	CC	+	+
A5	CC	-	+
A6	CC	+	-
C1	AA	-	+
C2	AA	-	-
C3	CA	-	+

A: Adult patients, C: Children patients; *: relapsed patient (8 years). Staining absent (-);Staining present (+).

It was observed that from the three children studied, two were homozygous AA and one was carrier for this allele. However, among the adults the prevalent genotype was homozygous CC and all patients were allele C carriers.

The immunohistochemistry study (Figure 2) showed that FOXP3 was stained at tumor cells, although the expression was not strong. The expression of CXCR4 was found in only two patients. Some samples showed FOXP3 and CXCR4 expression for both proteins in tumor cells. Interestingly, one adult patient, that had relapsed after eight years at diagnosis, showed tumor cells stained for CXCR4 and FOXP3 proteins.

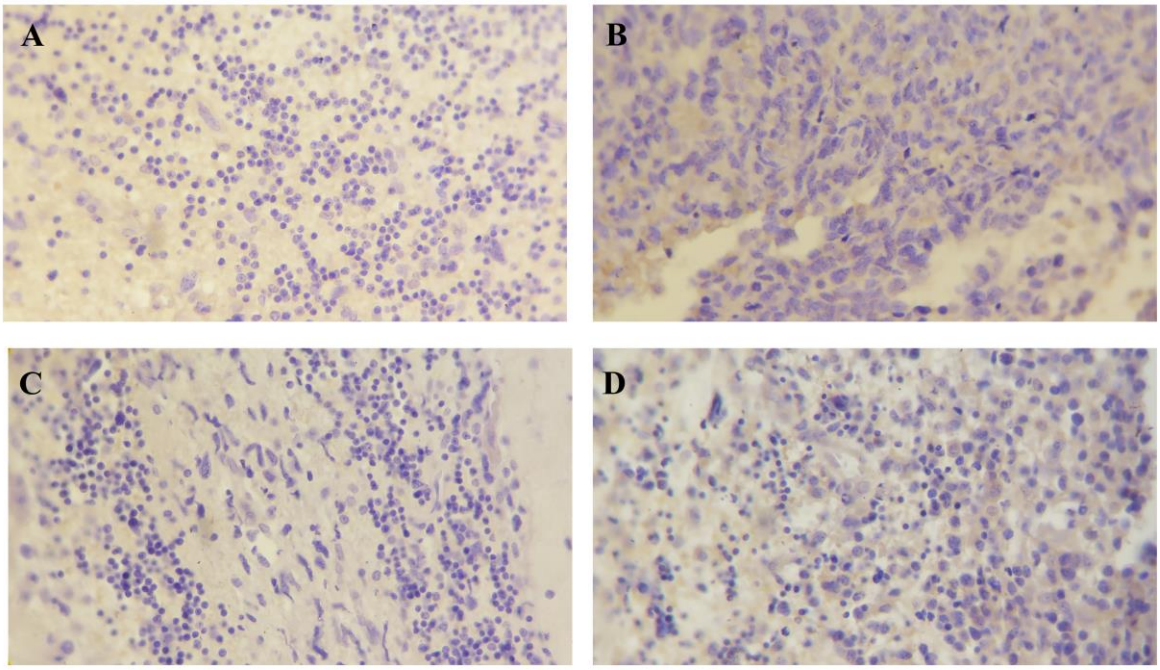

Figure 2. Immunohistochemistry staining for CXCR4 and FOXP3 in normal and tumoral tissue from MB patients. A: normal adjacent tissue without staining for CXCR4. **B:** tumoral tissue stained for CXCR4. **C**: normal tissue without staining for FOXP3. **D**: tumoral tissue stained for FOXP3.

DISCUSSION

MB is described to be more frequent in childhood and rarely seen at adult age (BRANDES, PALMISANO & MONFARDINI, 1999), however, our samples following more than 20 years, included more adults than children. The children incidence peaks was at 5–9 years old and among all samples approximately 70% of the patients were diagnosed before 20 years old. Interestingly, there is a slight increase in the incidence between 20–24 years, and the tumor is extremely rare after the fourth decade.

Among the three children studied, there were two AA rare homozygous and one carrier for this allele, while in adults, CC homozygous was the prevalent genotype and all patients were allele C carriers. Although a reduced numbers of samples were evaluated in this study, the authors suggest that the presence of the rare allele A in all children with MB deserves better investigation, once this polymorphism may be associated with risk for

determined age onset of MB.

There are controversial data about FOXP3 tumor expression role in cancer. DOUGLASS *et al.* (2014) showed that FOXP3 acts as an important tumor suppressor in breast cancer. However, other study showed that this protein may be involved in metastasis and tumor evasion in neuroblastoma, through modulation of CXCR4 expression (SUN *et al.*, 2007). Our findings indicated that the FOXP3 was expressed in six samples including adults and children, with no differences in protein expression between samples. Thus, as happens with other types of tumors, the role of FOXP3 in MB remains unknown and the distinction between adult and pediatric MB also remains unclear, as described by NAZMY *et al.* (2014).

The chemokine receptor CXCR4 and its ligand CXCL12 have been described to be involved in normal cerebellar development (REISS *et al.*, 2002; ZHU *et al.*, 2002) and several studies have shown the importance of CXCR4 in MB development and prognosis (SENGUPTA *et al.*, 2012; YUAN *et al.*, 2013). Our study indeed verified the presence of this protein in tumor cells from two individuals. However, more data are needed to inform if this protein expression is related to MB susceptibility and prognosis.

Albeit it was not possible to address the role of these proteins in medulloblastoma, this study showed for the first time the expression of FOXP3 in medulloblastoma tumor cells and also the genotype distribution of FOXP3 polymorphism rs3761548 among adults and children. It was observed the presence of CXCR4 protein in two samples. Additional studies adopting a bigger sample and collecting more information data could give a better understanding of these proteins in medulloblastoma microenvironment.

AUTHOR CONTRIBUTIONS

All authors were involved with experimental protocols and the writing of this paper and gave final approval for the submitted and published versions.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest

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5.4 ARTIGO 4

Genotype analyze of *FOXP3* and *CXCL12* polymorphisms and protein expression of FOXP3 and CXCR4 and susceptibility to Wilms' tumor

ABSTRACT

Wilms' tumor (WT) is an embrionary neoplasia that accounts for approximately 6% of all childhood tumors. The chemokine CXCL12 and its ligand CXCR4 are involved in the development of several organs, including kidney, and also is associated with tumor growth and metastasis. The transcription factor FOXP3 is expressed in several cell types, and polymorphisms in this gene are associated with the development of several diseases, like IPEX, diabetes type I and breast cancer. Thus, the aim of this study was to verify the genotype frequencies of *FOXP3* rs3761548 and rs2232365 polymorphisms and its protein expression, as well as evaluate the genotype frequency of *CXCL12* 1801157 polymorphism along with its receptor CXCR4 protein expression in WT. We also compare the results obtained from genetic and protein analysis with pathological data obtained from patient's reports. The patient's mean age at diagnosis was 3.75 years old. The case control study indicated a significant association for allele A carrier of *CXCL12* polymorphism rs1801157 with WT susceptibility (OR=5.261; 95% CI: 2.156 to 12.84; p=0.0002). The opposite was observed for allele G carriers of *FOXP3* polymorphism rs2232365 when it was analyzed male individuals (OR: 0.1164; 95% CI: 0.0227 to 0.5954; p=0.0091), or when it was analyzed in combination, male and female subjects (OR=0.1304; 95% CI: 0.05013 to 0.3394; p<0.0001), and no association was verified for *FOXP3* polymorphism rs3761548. It was not observed any association between FOXP3 polymorphisms and its protein expression in tumor and normal cells. The majority part of tumors and normal cells expressed FOXP3 and CXCR4 in the cytoplasm, although it was not correlated with age, gender, tumor size or the polymorphisms analyzed. These results suggest that these two genes could be used, after further studies, as prognostic markers to WT susceptibility.

Keywords: Wilms' Tumor, FOXP3, CXCL12, CXCR4, genetic polymorphism, protein expression.

INTRODUCTION

The Wilms' tumor (WT) develops from remnants of immature kidney. It is characterized as an embrionary tumor, composed of persistent blastema, dysplastic tubules, and supporting mesenchyme or stroma (BECKWITH, 1994). It accounts for approximately 6% of all childhood tumors (RIES *et al.*, 2004) and its incidence corresponds to 1 each 10 thousand children, usually unilateral and sporadic, with only 1% being considered hereditary (BRESLOW *et al.*, 2006).

Childhood cancers have differences from adult tumors, once they have different primary and histologic origins and also clinical outcomes, which suggest they have to be studied apart from adult malignant neoplasias (MALKIN, 1997). Besides, their early diagnosis age suggest a low exposition to risk factors, showing that genetic alterations possibly have more influence in the formation of these tumors when compared to adult tumors (DAVIDOFF, 2012).

The crosstalk among tumor microenvironment components influences the growth, invasiveness and metastatic process of tumor cells (YAQUB & AANDAHL, 2009), becoming the understanding of these complex networks of extremely importance for the discovery of prognostic markers and the development of new therapeutic strategies (FRIDMAN *et al.*, 2011).

Chemokines are known to play a major role in several homeostatic (VIOLA & LUSTER, 2008), pathological (BALKWILL, 2003) and developmental process (KLEIN *et al.*, 2001). Among them, CXCL12 and its receptor CXCR4 appear to be involved with the development of several organs (NAGASAWA, TACHIBANA & KISHIMOTO, 1998; SALCEDO & OPPENHEIM, 2003), including kidney (TAKABATAKE *et al.*, 2009), and also are related to tumor growth (ORIMO *et al.*, 2005) and metastatic process in many types of cancer (MULLER *et al.*, 2001).

Polymorphisms in *CXCL12* gene regulatory regions, like the rs1801157, can change the protein expression and may be associated with susceptibility to certain diseases (WINKLER *et al.*, 1998). Several studies have been made to verify the genotypic frequency of *CXCL12* polymorphism rs1801157 in different populations in order to address its possible role in tumor pathogenesis (DE OLIVEIRA *et al.*, 2007; DE OLIVEIRA *et al.*, 2009; DE LOURDES PERIM *et al.*, 2013; DE OLIVEIRA *et al.*, 2013).

The Forkhead box protein (FOXP3) is a transcription factor that plays a major role on regulation and development of immune system (HORI, NOMURA & SAKAGUCHI, 2003;

COFFER & BURGERING, 2004). Although it was first described to be restricted to hematopoietic lineages, recent studies have shown FOXP3 expression in several tissues, including tumoral cells (ZUO *et al.*, 2007; EBERT *et al.*, 2008; KARANIKAS *et al.*, 2008), and also has suggested that its localization can be nuclear or cytoplasmic, which can be related with patients prognostic (TAKENAKA *et al.*, 2013).

Several diseases have been associated with FOXP3 dysfunction, like the immune dysregulation, polyendocrinopathy, enteropathy, X linked syndrome (IPEX) (VAN DER VLIET & NIEUWENHUIS, 2007), type 1 diabetes (BASSUNY *et al.*, 2003), and thyroid autoimmunity (BAN *et al.*, 2007). Genetic analysis of some diseases like psoriasis (GAO *et al.*, 2010) and breast cancer showed association with the single nucleotide polymorphisms (SNPs) rs376154 (-3279 C/A) and the rs2232365 (-924 A/G) was described to be associated with vitiligo (SONG *et al.*, 2013). The study of these allelic variants can elucidate the role of these polymorphisms on gene transcription regulation in several pathologies, including cancer.

Therefore, the aim of this study was to verify the genotype frequencies of *FOXP3* rs3761548 and rs2232365 polymorphisms and its protein expression, as well as evaluate the genotype frequency of *CXCL12* 1801157 polymorphism along with its receptor CXCR4 protein expression in WT. We also compare the results obtained from genetic and protein analysis with pathological data obtained from patients reports.

MATERIALS AND METHODS

Human Subjects

A total of 32 samples of paraffin embedded tissues containing normal and tumor cells from a 23 years retrospective study were obtained at University Hospital of the State University of Londrina, Londrina, Paraná State, Brazil. Pathological data (age, tumor size and gender) were collected from pathology reports obtained at the same institution. For control group, blood samples from 78 healthy individuals neoplasia free were collected in the same institution. The controls were predominantly Caucasian, a prevalent population in Southern Brazil due to European colonization.

The protocol was approved by the Institutional Human Research Ethics Committee of the State University of Londrina, Paraná, Brazil (Nº.171231134.0000.5231). The children's parents signed an informed consent for their children participation in this study. The chief of

Department of Pathology, Clinical and Toxicological Analysis at the University Hospital of State University of Londrina, signed an authorization for the use of paraffin blocks in this study.

DNA Extraction

In the tumor sample group, the genomic DNA was isolated from formalin-fixed paraffin embedded samples and extracted according to the protocol from innuPREP DNA Mini Kit (Analytik Jena AG, Jena, Germany), according to manufacturer's instructions. From neoplasia-free control group, the DNA was obtained from peripheral blood white cells using the genomic DNA extraction kit Mini Spin (Biometrix, Curitiba, PR, Brazil), according to manufacturer's instructions. The DNA was quantified by NanoDrop 2000® (NanoDrop Technologies, Wilmington, DE, USA) at a wavelength of 260 nm. The purity regarding protein contamination was evaluated by the ratio between the wavelength at 260 nm length and 280 nm.

Genotype of *CXCL12* genetic polymorphism rs1801157

Approximately 100 ng of DNA was amplified by RFLP polymerase chain reaction (PCR) with specific primers for *CXCL12* (Table 1) following the GenBank accession number L36033. The samples were amplified using dNTP 100 µM, 150 pM of each primer, $MgCl_2$ 1,5 mMm buffer 10% and 1.25 units of Taq polymerase (Invitrogen, Carlsbad, CA, USA). PCR conditions were 1 cycle of 5 min denaturation at 94°C, followed by 40 cycles of 1 min at 94°C, 1 min at 60°C, 1 min at 72°C, and 10 min elongation at 72°C in a thermocycler A200 Gradient Thermal Cycler (LongGene, Hangzhou, China). This polymorphism consists in a guanine to adenine change in the position 801 in the 3'UTR region. PCR products were subjected to restriction digestion by incubating with *Msp*I (10U) (Promega, Madison, WI, USA) during 4h at 37^{o}C.

Genotype of *FOXP3* genetic polymorphisms rs3761548 and rs2232365

Approximately 100 ng of DNA was amplified by allele specific PCR with specific primers for FOXP3 (Table 1) following the GenBank accession number NG_007392. The samples were amplified using dNTP 100 µM, 150 pM of each primer, $MgCl_2$ 1,5 mM, buffer

10% and 1.25 units of Taq polymerase (Invitrogen, Carlsbad, CA, USA). PCR conditions were 10 min denaturation at 94°C, followed by 40 cycles of 30s at 94°C, 30s at 67°C and 1 min at 72°C, and 10 min elongation at 72°C in a thermocycler A200 Gradient Thermal Cycler (LongGene, Hangzhou, China).

Table 1. Primers for polymorphism study of *FOXP3* and *CXCL12*

Polymorphism	Primers sequence	PCR product
rs3761548 (C/A) *(FOXP3)*	5'-CTGGCTCTCTCCCCAACTGA-3' 5'-ACAGAGCCCATCATCAGACTCTCTA-3'	Allele A 334bp
	5'-TGGCTCTCTCCCCAACTGC-3' 5'-ACAGAGCCCATCATCAGACTCTCTA-3'	Allele C 333bp
rs2232365 (A/G) *(FOXP3)*	5'-CCCAGCTCAAGAGACCCCA-3' 5'-GGGCTAGTGAGGAGGCTATTGTAAC-3'	Allele A 442bp
	5'-CCAGCTCAAGAGACCCCG-3' 5'-GCTATTGTAACAGTCCTGGCAAGTG-3'	Allele G 427bp
rs1801157 (G/A) *(CXCL12)*	5'-CAGTCAACCTGGGCAAAGCC-3' 5'-CCTGAGAGTCCTTTTGCGGG-3'	293bp

All PCRs products were analyzed by electrophoresis on polyacrylamide gel (10%) and detected using a silver staining method.

Immunohistochemical staining

The FOXP3 and CXCR4 status were analyzed immunohistochemically on formalin-fixed, paraffin-embedded tumor sections, using antibodies at a dilution of 1:25 and 1:100, respectively. 3 μm of tissue sections was obtained from WT samples and examined on a Silanized Slide (Starfrost, Knittel, Germany). Samples were heated at 56°C, deparaffinized in xylene, and rehydrated in a graded alcohol series. Antigen retrieval was performed with citrate/citric acid buffer and then, a mouse monoclonal antibody anti-human FOXP3 (clone 236A/E7; eBioscience, San Diego, CA, USA) and a mouse antibody anti-human (CD184) CXCR4 (clone polyclonal; eBioscience, San Diego, CA, USA) were used. The sections were stabilized at room temperature for 30 min and washed with PBS (phosphate buffered saline) and anti-mouse/rabbit HRP secondary antibody was used as second step (Bio SB Inc. Santa Barbara, CA, USA). The diaminobenzidine (DAB) chromogen system was used (Bio SB Inc. Santa Barbara, CA, USA) and counter staining was performed with Gill's hematoxylin and the slides were completed setting the coverslips using Canada balsam. Controls were performed to verify the specificity of primary antibody and all analyses were made by

experienced pathologists, who were blinded to the clinicopathological data. The FOXP3 and CXCR4 staining within the tumor cell cytoplasm and nuclei was scored as absent or present.

Statistical analysis

The case-control study for TW susceptibility was assessed through Odds ratio (OR) analysis, adopting a 95% confidence interval (CI), and Fisher's exact test, performed using Prism 6 for Windows (GraphPad Software, San Diego, CA, USA). The *FOXP3* polymorphisms analysis were performed apart for females and males, once this gene is localized at X chromosome and only females can be considered heterozygous, while males are considered hemizygous. For the analysis of the immunohistochemistry and genetic polymorphisms results in relation to pathological characteristics, we adopt the Chi square statistical test, performed using SPSS Statistics 20.0 software (SPSS inc., Chicago, IL, USA). The *p* value was considered statistically significant when *p* value <0.05.

RESULTS

Genetic Polymorphism Analysis

The electrophoretic profile for the *CXCL12* polymorphism rs1801157 are represented in figures 1A and B. Figure 1A shows the 293 bp amplicons of *CXCL12* gene. The *Msp*I enzyme cut the amplicons that have the restrict site preserved, which are those without mutation. Thus, the *CXCL12* GG genotype produces 100 bp and 193 bp products, the AA genotype produce a 293 base pair and the heterozygote genotype GA produces the three fragment sizes (Figure 1B).

The genotype frequency observed for *CXCL12* polymorphism for patients and controls was: 31,3% (10/32) and 70,5% (55/78) for GG homozygote; 56,2% (18/32) and 26,9% (21/78) for GA heterozygote; 12,5% (4/32) and 2,6% (2/78) for AA homozygote (Figure 1C).

The case control study indicated a positive association for allele A carrier from *CXCL12* polymorphism in relation to WT susceptibility (OR=5.261; 95% CI: 2.156 to 12.84; p=0.0002) (Figure 1D).

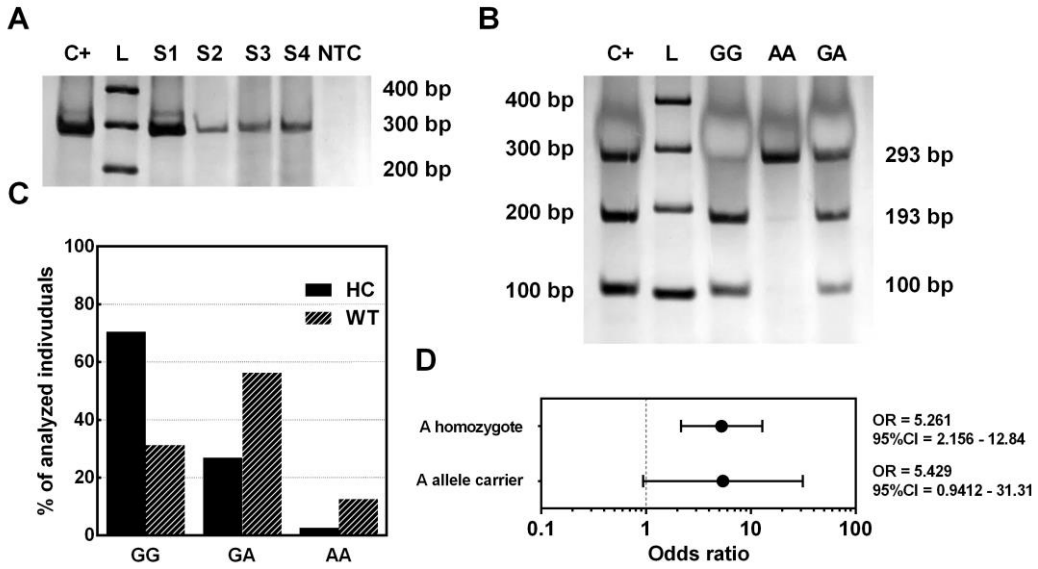

Figure 1. Analyse of *CXCL12* polymorphism rs1801157. A: Electrophoretic profile of *CXCL12* polymorphism rs1801157. C+: positive control; L: Ladder 100 bp; S1, S2, S3, S4: samples; NTC: no template control. B: Electrophoretic profile after digestion with *Msp*I. L: Ladder 100 bp; GG: Wild type homozygote individuals; GA: Heterozygote individuals; AA: Mutant homozygote individuals. C. Genotype frequency of HC (healthy control) and WT (Wilms' Tumor) individuals. D. Odds ratio analysis.

The electrophoretic profile for *FOXP3* polymorphism rs3761548 can be observed in Figure 2A. Genotype frequency of *FOXP3* polymorphism rs3761548 for female patients and controls was: 47.4% (9/19) and 54.1% (20/37) for CC homozygote, 26.3% (5/19) and 13.5% (5/37) for CA heterozygote and 26.3% (5/19) and 32.4% (12/37) for AA homozygote, respectively. Male patient's and control's genotype frequency for this polymorphism was: 69.2% (9/13) and 61.0% (25/41) for C hemizygote and 30.8% (4/13) and 39.0% (16/41) for A hemizygote (Figure 2B).

For the *FOXP3* polymorphism rs3761548 the case control study indicated no association between this polymorphism genotypes and susceptibility to WT (OR:1.061; 95% CI: 0.4622 to 2.434; p=1.0000) (Figure 2C).

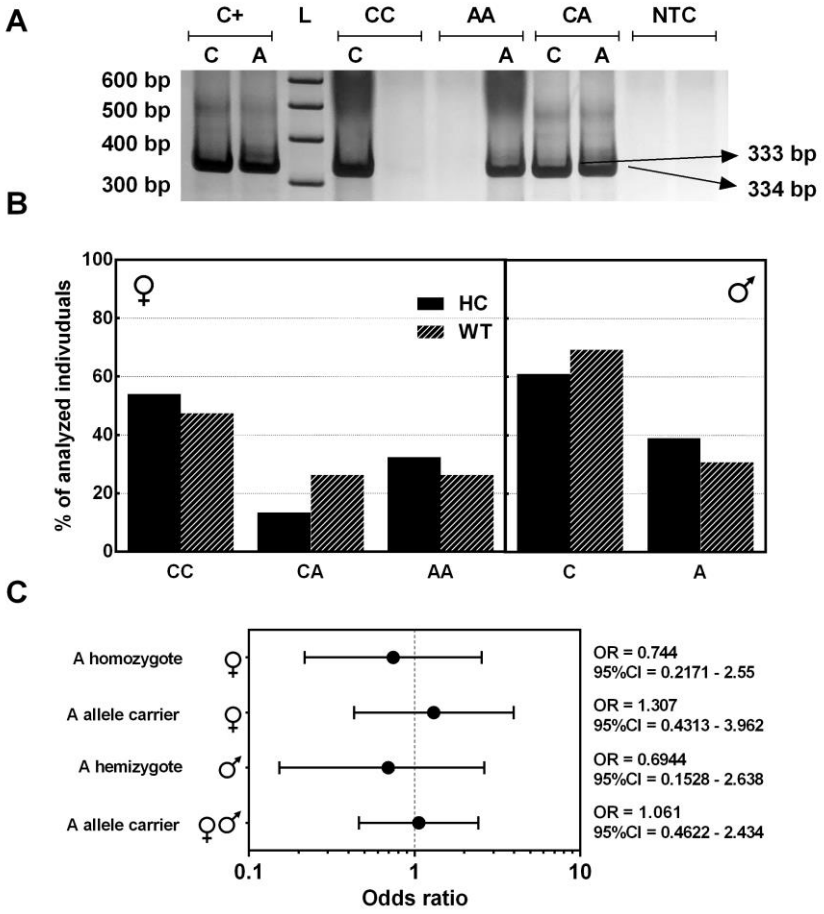

Figure 2. Analyse of *FOXP3* polymorphism rs3761548. A: Electrophoretic profile. C+: positive control; L: Ladder 100 bp; CC: Wild type homozygote individual; CA: Heterozygote individual; AA: Mutant homozygote individual; NTC: no template control. B: Genotype frequency of HC (healthy control) and WT (Wilms' Tumor) individuals, in female individuals (left) and male individuals (right). C. Odds ratio analysis.

The electrophoretic profile for *FOXP3* rs2232365 polymorphism is represented in Figure 3A. The genotype frequency observed was 63,2% (12/19) and 37.8% (14/37) for AA homozygote, 21.0% (4/19) and 43.3% (16/37) for AG heterozygote, and 15.8% (3/19) and 18.9% (7/37) for GG homozygote, for female patients and controls, respectively. The genotype frequencies for male patients and controls were, respectively: 85.6% (11/13) and 39.0% (16/41) for A hemizygote and 15.4% (2/13) and 61.0% (25/41) for G hemizygote (Figure 3B).

Unlike the other *FOXP3* polymorphism analyzed, the case control study indicated that the allele G carrier of *FOXP3* polymorphism rs2232365, conferred protection against WT development, when it was analyzed males individuals (OR: 0.1164; 95% CI: 0.0227 to 0.5954; p=0.0091), or when it was analyzed in combination, male and female subjects (OR=0.1304; 95% CI: 0.05013 to 0.3394; p<0.0001) (Figure 3C).

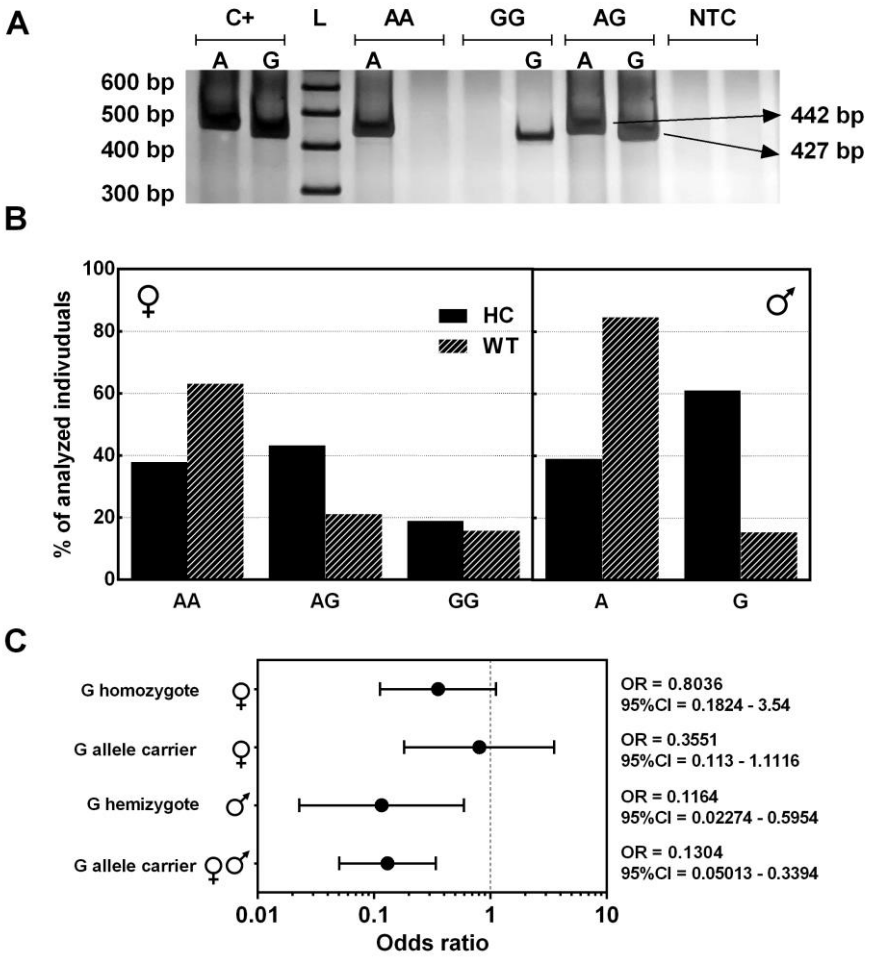

Figure 3. Analyse of *FOXP3* polymorphism rs2232365. A: Electrophoretic profile. C+: positive control; L: Ladder 100 bp ; AA: Wild type homozygote individual; GA: Heterozygote individual; GG: Mutant homozygote individual; NTC: no template control. B: Genotype frequency of HC (healthy control) and WT (Wilms' Tumor) individuals, in female individuals (left) and male individuals (right). C. Odds ratio analysis.

The table 2 displays the clinical characteristics analyzed for WT samples.

Table 2. Clinical characteristics from WT patients.

Clinical characteristics		
Gender	Male	Female
	N=13	N=19
Age	mean	range
	3.75	1 - 8 anos
Tumor size	≤ 8 cm	> 8 cm
	N=7	N=18

When it was compared the polymorphism genotypes with these clinical characteristics, we observed that all patients who were AA mutant homozygous for *FOXP3* rs3761548

polymorphism had tumor size higher than 8 cm (p=0.041). The AA homozygous genotype for *FOXP3* rs2232365 polymorphism was more observed in tumors higher than 8cm (p=0.011).

Immunohistochemistry Analysis

The FOXP3 and CXCR4 staining are described in table 3, in relation to tumor cells.

Table 3. FOXP3 and CXCR4 staining in tumor cells.

		FOXP3 (n=29)		CXCR4 (n=30)	
		Citoplasmic	Nuclear	Citoplasmic	Nuclear
Tumor cells	Absent	9	26	9	29
	Present	20	3	21	1

When we analyzed FOXP3 tumor expression we observed that the majority expressed this protein in the cytoplasm (Table 3), although it was not correlated with any pathological characteristic like age, gender and tumor size. Figure 4 shows the FOXP3 staining in normal adjacent tissue and tumor cells.

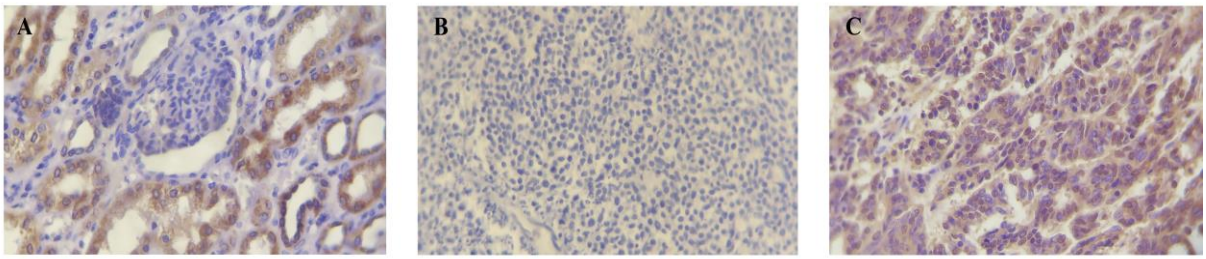

Figure 4. FOXP3 expression by immunohistochemistry in normal and tumor cells from WT patients. A: normal adjacent tissue stained for FOXP3. B: tumor cells without staining. C: tumor cells stained. Magnification 400x.

For CXCR4 cytoplasmic staining we analyzed 31 individuals and we observed that 22 of them showed CXCR4 staining (Figure 5), although it was not correlated with any pathological characteristic, like age, tumor size and gender. When we compared FOXP3 and CXCR4 staining we did not verify any statistically significant correlation among CXCR4 cytoplasmic expression with FOXP3 nuclear expression (p=0.203), and neither when it was compared with FOXP3 cytoplasmic expression (p=0.064).

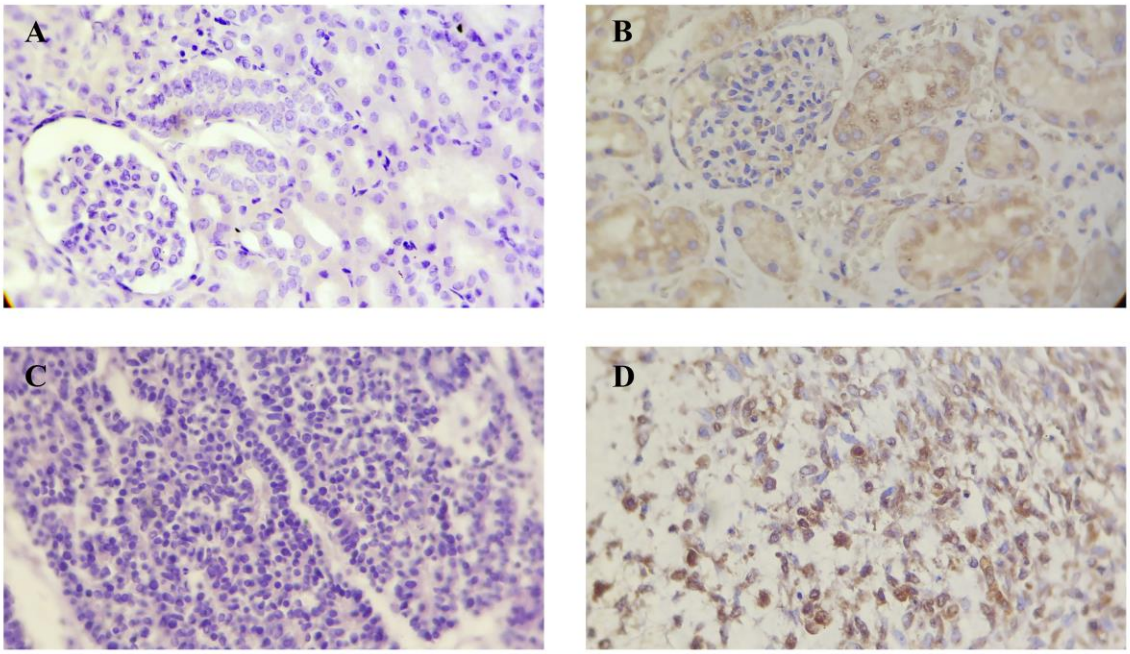

Figure 5. CXCR4 expression by immunohistochemistry in normal and tumor cells. A: normal adjacent tissue without staining; B: normal adjacent tissue stained; C: tumor tissue without staining; D: tumor tissue stained. Magnification 400x.

We did not verify any association of the *CXCL12* polymorphism with CXCR4 expression. It was not observed any association between *FOXP3* polymorphisms and its protein expression in tumor and normal cells. However, it was observed that individuals homozygous GG for the *CXCL12* polymorphism rs1801157 showed nuclear staining of FOXP3 protein (p=0,042).

It was possible to analyze the normal adjacent tissue of 21 individuals, and all of them expressed FOXP3 in different levels (Table 4), although it was not verified any statistically difference between normal and tumor FOXP3 expression. The CXCR4 protein was expressed in 16 individuals, and there were no association between normal and tumor expression of this protein.

DISCUSSION

In this study we analyzed 32 tissue samples from patients diagnosed with WT between January 1990 and December 2013. The overall mean ages found were 45 months, which is in accordance with Breslow and coworkers observations (BRESLOW *et al.*, 2006).

Studies have shown that WT cells express markers of early kidney development (LI *et al.*, 2002; LI, KESSLER & WILLIAMS, 2005). In addition, several studies have highlighted the presence and importance of CXCL12 and CXCR4 during kidney development (GRONE *et al.*, 2002; DING *et al.*, 2006; TAKABATAKE *et al.*, 2009; UELAND *et al.*, 2009). In this context we investigate the influence of *CXCL12* polymorphism rs1801157, a genetic variant that had already been associated with susceptibility to other childhood cancer (DE LOURDES PERIM *et al.*, 2013), and we verified a strong positive association for allele A carrier with WT susceptibility (OR=6.757; 95% IC: 2.585 to 17.66).

The *CXCL12* polymorphism rs1801157 is present in a regulatory region of *CXCL12* gene, and it was showed that allele A carriers had altered levels of CXCL12 mRNA, compared with GG carriers (DE OLIVEIRA *et al.*, 2013). It was observed that spatial and temporal relationship between CXCL12 and CXCR4 positive cells are required for a regular kidney development (TAKABATAKE *et al.*, 2009). Thereby, the authors suggest that allele A carriers, that possibly present alter levels of CXCL12, which can cause a lower expression of this chemokine, can be more susceptible to alterations in kidney once CXCL12 regular expression is required for its development.

The FOXP3 protein is a transcription factor that has different expression patterns in a great variety of cell types, and its role in cancer cells remains unclear. Nowadays it is well established that this protein can be expressed by different cell types, aside of its expression in Tregs, which include normal (ZUO *et al.*, 2007) and tumor (HINZ *et al.*, 2007; KARANIKAS *et al.*, 2008) cells. Studies have supported that FOXP3 protein also has different roles, acting as a tumor suppressor protein (ZUO *et al.*, 2007), or as evading mechanisms for tumors, when expressed in Tregs (ZOU, 2006), or in the cytoplasm or nucleus of tumor cells (MERLO *et al.*, 2009).

It is known that polymorphisms present in the regulatory region of a gene could affect its protein expression, however in this study we did not find any statistical significant alterations in FOXP3 protein expression in tumor cells when compared with both polymorphisms of *FOXP3*. Although AA homozygotes for *FOXP3* polymorphism rs3761548 have been considered a susceptibility maker to triple negative breast cancer (LOPES *et al.*, 2014), we did not find any association for AA homozygotes or allele A carriers with WT susceptibility. Nevertheless, we observed that allele G carrier of *FOXP3* polymorphism rs2232365 conferred protection against WT development, when it was analyzed on males (OR: 0.1091; 95% CI: 0.2122 to 0.5609), or when it was analyzed combined, on male and female subjects (OR=0.2270; 95% CI: 0.09261 to 0.5562). This result suggests that the

transcription factor FOXP3 plays an important role in WT development and that this polymorphism somehow could influence the FOXP3 function, which could change the kidney microenvironment, through regulation of genes, in order to evade the development of this tumor.

The real function of these polymorphisms in *FOXP3* gene is unknown. However, it is possible that these two polymorphisms in *FOXP3* regulatory regions act in different ways in WT, once we observed that one of them was associated with protection and the other showed no association. Besides, we observed an interesting data: individuals homozygous for mutant allele A for *FOXP3* polymorphism rs3761548 and individuals homozygous for the wild type allele A for the *FOXP3* polymorphism rs2232365 presented tumor sized bigger than 8 cm.

Among other FOXP3 actions, it was observed that it can act influencing the expression of chemokine receptors, like CXCR4 (DOUGLASS *et al.*, 2014). However, this was not observed in WT patients when it was compared CXCR4 cytoplasmic expression with FOXP3 nuclear expression (p=0.203), and neither when it was compared with FOXP3 cytoplasmic expression (p=0.064), although the FOXP3 nuclear expression has been associated with individuals homozygous GG for the *CXCL12* polymorphism rs1801157 (p=0,042), that is the ligand of this chemokine receptor.

CONCLUSION

Our study showed for the first time the presence of FOXP3 in tumor and normal cells of WT patients and also verified the expression of CXCR4 in two samples. Besides, our case control study demonstrate that allele G carrier of *FOXP3* polymorphism rs2232365 confered protection against WT susceptibility and allele A carrier of *CXCL12* polymorphism rs1801157, on the other hand, are positively associated with WT susceptibility. Although further studies are needed to characterize the role of these two genes in WT development, it is possible to suggest that these genes can be used as prognostic markers to WT susceptibility in the future.

AUTHOR CONTRIBUTIONS

All authors were involved with experimental protocols and in writing the paper and gave final approval for the submitted and published versions.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest

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6 CONCLUSÃO

- ✓ Foram encontrados 88 casos de tumores pediátricos sólidos na região de Londrina, PR, em um estudo retrospectivo de Janeiro de 1990 a Dezembro de 2013. Os tumores mais frequentes observados neste estudo foram: TW (43,182%), NB (39,773%), ACT (10,227%) e MB (6,818%).

- ✓ A idade média dos pacientes com TW foi 3,51 anos e a razão menino/menina 0,73. A idade média dos pacientes com NB foi 3,48 anos, com aproximadamente 74% dos casos acontecendo abaixo de 4 anos de idade e possuindo uma razão de 1,18 entre meninos e meninas. Os pacientes com MB possuíam uma idade média de 6,39 ao diagnóstico, afetando ambos os sexos igualmente. Já para os pacientes com tumor na ACT a idade média foi de 8,29, com quase 45% acontecendo abaixo dos 4 anos de idade, possuindo uma razão menino/menina de 0,50.

- ✓ Nosso estudo foi o primeiro a investigar e constatar a presença das proteínas FOXP3 em células tumorais de MB, embora a expressão tenha sido baixa. Nós observamos também a presença de CXCR4 em dois pacientes.

- ✓ Foi observado que a maioria dos pacientes infantis com MB eram homozigotos AA, enquanto os adultos eram prevalentemente homozigotos CC, com todos eles sendo pelo menos portadores do alelo C para o polimorfismo rs3761548 do gene *FOXP3.*

- ✓ Nosso estudo foi o primeiro também a investigar e constatar a presença das proteínas FOXP3 e CXCR4 em células normais e tumorais de pacientes diagnosticados com TW.

- ✓ Os estudos caso controle demonstraram uma associação positiva do portador do alelo G, do polimorfismo rs2232365 do gene *FOXP3* e do portador do alelo A do polimorfismo rs1801157 do gene *CXCL12* com a susceptibilidade ao desenvolvimento do TW.

7 ANEXOS

Universidade Estadual de Londrina

PARANÁ
GOVERNO DO ESTADO

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

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

Prezado(a) Senhor(a):

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

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

Situação do Projeto: **Aprovado**

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

Londrina, 30 de setembro de 2013.

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

Comitê de Ética em Pesquisa Envolvendo Seres Humanos - UEL