BRUNA KARINA BANIN HIRATA

ANÁLISE DE POLIMORFISMOS GENÉTICOS, EXPRESSÃO GÊNICA E METILAÇÃO DA REGIÃO PROMOTORA DO FATOR DE TRANSCRIÇÃO FOXP3:

IMPLICAÇÕES NA PATOGÊNESE DE SUBTIPOS AGRESSIVOS DO CÂNCER DE MAMA

Londrina
2016

BRUNA KARINA BANIN HIRATA

ANÁLISE DE POLIMORFISMOS GENÉTICOS, EXPRESSÃO GÊNICA E METILAÇÃO DA REGIÃO PROMOTORA DO FATOR DE TRANSCRIÇÃO FOXP3: IMPLICAÇÕES NA PATOGÊNESE DE SUBTIPOS AGRESSIVOS DO CÂNCER DE MAMA

Tese apresentada ao Programa de Pós-Graduação em Patologia Experimental da Universidade Estadual de Londrina, como requisito parcial à obtenção do título de Doutora.

Orientadora: Profa. Dra. Maria Angelica Ehara Watanabe

Co-orientadora: Profa. Dra. Karen Brajão de Oliveira

Londrina
2016

Ficha de identificação da obra elaborada pelo autor, através do Programa de Geração Automática do Sistema de Bibliotecas da UEL

Hirata, Bruna Karina Banin.

Análise de polimorfismos genéticos, expressão gênica e metilação da região promotora do fator de transcrição FOXP3: implicações na patogênese de subtipos agressivos do câncer de mama / Bruna Karina Banin Hirata. - Londrina, 2016.
107 f.

Orientador: Maria Angelica Ehara Watanabe.
Tese (Doutorado em Patologia Experimental) - Universidade Estadual de Londrina, Centro de Ciências Biológicas, Programa de Pós-Graduação em Patologia Experimental, 2016.
Inclui bibliografia.

1. Câncer de mama - Tese. 2. Células T reguladoras - Tese. 3. FOXP3 - Tese. 4. Polimorfismo genético - Tese. I. Watanabe, Maria Angelica Ehara. II. Universidade Estadual de Londrina. Centro de Ciências Biológicas. Programa de Pós-Graduação em Patologia Experimental. III. Título.

BRUNA KARINA BANIN HIRATA

ANÁLISE DE POLIMORFISMOS GENÉTICOS, EXPRESSÃO GÊNICA E METILAÇÃO DA REGIÃO PROMOTORA DO FATOR DE TRANSCRIÇÃO FOXP3:

IMPLICAÇÕES NA PATOGÊNESE DE SUBTIPOS AGRESSIVOS DO CÂNCER DE MAMA

BANCA EXAMINADORA

_____
Profa. Dra. Maria Angelica Ehara Watanabe
Universidade Estadual de Londrina - UEL

_____
Profa. Dra. Julie Massayo Maeda Oda
Universidade Federal do Mato Grosso do Sul - UEMS

_____
Profa. Dra. Marla Karine Amarante
Universidade Estadual de Londrina - UEL

_____
Profa. Dra. Roberta Losi Guembarovski
Universidade Estadual de Londrina - UEL

_____
Profa. Dra. Vanessa Jacob Victorino
Universidade Estadual de Londrina - UEL

Londrina, 16 de Dezembro de 2016.

"Dedico este trabalho à minha mãe Celia Regina Banin, minha avó Benedita Rondão Banin e à meu noivo Carlos Renato Cortez Lopes por todo o apoio e carinho que foram essenciais para eu prosseguir essa jornada"

AGRADECIMENTOS

À minha orientadora, **Profa. Dra. Maria Angelica Ehara Watanabe**, pela excelente orientação que foi crucial para o desenvolvimento deste trabalho, pela dedicação e pelo exemplo de profissional. Acima de tudo agradeço por ser mais que uma orientadora ser uma amiga e praticamente uma mãe, me incentivando e me ajudando sempre. É um privilégio tê-la como orientadora. Obrigada por tudo!

À minha co-orientadora, **Profa. Dra. Karen Brajão de Oliveira**, que me auxiliou no desenvolvimento e na correção deste trabalho sendo sempre muito solícita e disposta a ajudar.

Aos amigos de laboratório: **Alberto Yoichi Sakaguchi, Carlos Eduardo Coral de Oliveira, Carolina Batista Ariza, Cintya Mayumi Ishibashi, Diego Lima Petenuci, Glauco Akelinghton F. Vitiello, Marcos Henrique Rosa, Marla Karine Amarante, Mayara Tiemi Enokida, Nathalia de Souza Pereira, Thiago Cezar Fujita e Vânia Darc de Castro,** com os quais tenho passado a maior parte dos meus dias e posso dizer que são para mim como uma família, pela qual tenho uma imensa consideração. Agradeço pela contribuição no desenvolvimento deste trabalho e pela amizade!

Aos membros da banca examinadora, **Profa. Dra. Julie Massayo Maeda Oda, Profa. Dra. Marla Karine Amarante, Profa. Dra. Roberta Losi Guembarovski e Profa. Dra. Vanessa Jacob Victorino**, pelo aceite em avaliar o trabalho e pela colaboração com a melhora deste trabalho.

Aos médicos oncologistas do Hospital do Câncer de Londrina **Clodoaldo Zago Campos e Walter Jorge Sobrinho** que colaboraram com a obtenção das amostras, e às médicas patologistas **Alda Losi Guembarovski, Daniela Rudgeri Derossi e Marina Okuyama Kishima** pela inestimável ajuda na análise das amostras e na leitura das lâminas de imunohistoquímica.

Agradeço à equipe do Ambulatório de Especialidades do Hospital Universitário (AEHU) que auxiliaram na coleta de amostras utilizadas no presente estudo, à equipe do setor de prontuários do Hospital do Câncer de Londrina pelo auxílio na pesquisa de informações

clínicas das pacientes e à técnica de laboratório **Maria Lucia Camillo de Almeida** do Hospital Universitário que auxiliou na realização da técnica de imunohistoquímica.

A **Universidade Estadual de Londrina** que fez possível a realização deste trabalho.

Ao **Programa de Pós-Graduação em Patologia Experimental** pela formação acadêmica e científica.

A **todas as doadoras voluntárias** que cederam amostras biológicas, contribuindo de maneira ímpar, e sem as quais seria impossível o desenvolvimento deste trabalho, muito obrigada!

Às agências de fomento a pesquisa que financiaram este projeto: CNPq, CAPES, Fundação Araucária e FAEPE-UEL.

Aos amigos que tornaram essa jornada mais fácil, especialmente à **Julie Massayo Maeda Oda** e **Patricia Midori Murobushi Ozawa,** que mesmo longe sempre se fizeram presente. Obrigada pela amizade, conselhos, apoio e torcida! Esta amizade, tão especial, levarei para sempre!

A todos os membros da minha **família**, agradeço pela torcida sincera, apoio constante, orações e pelo carinho que recebo mesmo quando nos separamos por alguns quilômetros. Cada um tem uma importância inestimável para mim, muito obrigada! Amo vocês!

A **Rosa Maria Cortez**, **Luiz Carlos Lopes**, **Luiz Felipe C. Lopes**, **Ana Carolina C. Lopes** e **Rafael G. Campos** agradeço por me considerarem parte da família, constituindo o meu porto-seguro aqui em Londrina e sem os quais esta trajetória teria sido mais difícil. Obrigada pelo apoio e pelo carinho imenso! Amo vocês!

Especialmente, à minha mãe **Célia Regina Banin** e minha avó **Benedita Rondão Banin**, que foram peças chaves na construção do que eu sou hoje. Agradeço pela torcida, conselhos, apoio incondicional e carinho inestimável. Tudo o que fizeram por mim foi valioso e com certeza sem vocês não teria chegado tão longe! Amo vocês!

Ao meu noivo **Carlos Renato Cortez Lopes**, por ser o melhor companheiro que alguém poderia ter. Agradeço pelo apoio, compreensão, motivação, torcida e por cuidar de mim. Obrigada pelo carinho e por estar sempre ao meu lado, sem você teria sido muito mais difícil. Amo você!

À **Deus**, que me deu sabedoria e força para superar mais esta etapa da minha vida. Pelas bênçãos de cada dia, as quais muitos denominam sorte e eu denomino presença de Deus. Apesar do muito esforço necessário para chegar até aqui reconheço que nada seria possível se não fosse pelo poder Dele. Serei eternamente grata!

“É do buscar e não do achar que nasce o que eu não conhecia”

Clarice Lispector

BANIN-HIRATA, Bruna Karina. **Análise de polimorfismos genéticos, expressão gênica e metilação da região promotora do fator de transcrição FOXP3: implicações na patogênese de subtipos agressivos do câncer de mama.** 2016. 107p. Tese de Doutorado (Programa de Pós-graduação em Patologia Experimental) – Universidade Estadual de Londrina, Londrina, 2016.

RESUMO

O grau de heterogeneidade molecular e celular no câncer de mama e o grande número de eventos genéticos envolvidos no controle do crescimento celular, diferenciação, proliferação e metástase dependem da interação tumor-hospedeiro. O câncer de mama consiste em um microambiente complexo que inclui, além das células neoplásicas e o estroma circundante, células do sistema imunológico. As células T reguladoras (Tregs) são importantes na supressão de resposta imune efetora e desta forma podem contribuir para a tumorigênese. Inicialmente acreditava-se que a proteína *forkheadbox 3* (FOXP3) fosse exclusiva dessas células, no entanto alguns estudos têm encontrado expressão deste fator de transcrição também em células epiteliais, inclusive de mama, onde pode atuar como um gene supressor tumoral. Sabe-se que polimorfismos genéticos podem modificar funcionalmente ou quantitativamente o *FOXP3*. Por outro lado, estudos tem mostrado que a regulação epigenética, como a metilação do DNA, é crucial para o controle de expressão do locus *FOXP3*. O presente estudo teve por objetivo analisar as variantes alélicas g.10403A>G (rs2232365) e g.8048A>C (rs3761548) entre pacientes com câncer de mama e mulheres livres de neoplasia mamária, o perfil de metilação da região promotora do gene, bem como sua expressão gênica. O genótipo AA do polimorfismo g.10403A>G foi associado com maior chance de desenvolvimento do câncer de mama (p=0,046). Enquanto o genótipo GG foi correlacionado com maior Ki-67 (p=0,019) no subtipo HER2-superexpresso (HER2+) e estadiamento TNM mais avançado no subtipo triplo-negativo (TN) (p=0.032). O genótipo AA do polimorfismo g.8048A>C foi correlacionado com maior Ki-67 (p=0.018) e menor grau histológico no subtipo HER2+ (p=0.026). O haplótipo GA foi correlacionado com menor grau histológico (p=0.009) e maior Ki-67 (p=0.036) no subtipo HER2+ e maior estadiamento em TN (p=0.044), enquanto o haplótipo AC foi correlacionado com menor Ki-67 (p=0.005) e estadiamento (p=0.027), nos subtipos HER2+ e TN, respectivamente. Além disso, o haplótipo AC foi correlacionado com maior expressão de RNAm de *FOXP3* (p=0.039). O subtipo Luminal B HER2+ (LB) apresentou 2,6 vezes menos expressão de RNAm de *FOXP3* comparado ao controle, enquanto o TN e HER2+ apresentaram uma expressão ligeiramente maior (1,6 e 1,8 vezes, respectivamente). A expressão de RNAm de FOXP3 não foi correlacionada com os parâmetros prognósticos das pacientes. Não houve diferença do perfil de metilação entre os tumores benignos, carcinoma ductal *in situ* e invasivo (p=0.96), assim como não houve diferença entre o tecido tumoral e normal adjacente (p=0.63). O perfil de metilação também não foi correlacionada com a expressão de RNAm e proteica de FOXP3, bem como com parâmetros prognósticos. Foi observada correlação significativa entre a expressão proteica citoplasmática de FOXP3 em células tumorais de mama com maior comprometimento de linfonodos (p=0.01) e uma tendência de correlação entre o infiltrado de células mononucleares FOXP3-positivas com maior grau histológico (p=0.068). Nossos resultados sugerem que no tumor de mama o perfil de metilação de *FOXP3* não está unicamente relacionado com a regulação transcricional deste gene, podendo outros mecanismos estar envolvidos, como polimorfismos genéticos, uma vez que o haplótipo AC dos polimorfismos estudados mostrou correlação com a expressão gênica. Além disso, este

haplótipo foi correlacionado com menor índice de proliferação celular Ki67 e estadiamento, indicando que o RNAm de *FOXP3* aumentado no tumor pode conferir melhor prognóstico. No entanto, ao analisar a expressão proteica, observamos que quando localizado no citoplasma da célula tumoral o FOXP3 pode estar correlacionado à pior prognóstico, como comprometimento de linfonodos. Desta maneira, sua influência no prognóstico pode depender da sua localização, assim como do subtipo tumoral de mama.

Palavras-chave: Câncer de mama. FOXP3. Tregs. Polimorfismo genético. Metilação de DNA.

BANIN-HIRATA, Bruna Karina. **Analysis of genetic polymorphisms, genic expression and promoter methylation of FOXP3 transcription factor: implications in pathogenesis of aggressive breast cancer subtypes.** 2016. 107p. Doctoral Tesis (Post-Graduate Program in Experimental Pathology) – Londrina State University, Londrina, 2016.

ABSTRACT

The degree of molecular and cellular heterogeneity in breast cancer and the large number of molecular events involved in the control of cell growth, differentiation, proliferation and metastasis depend on the tumor-host interaction. The breast cancer is a complex microenvironment which includes, in addition to neoplastic cells and surrounding stroma the immune cells. The regulatory T cells (Tregs) are important in suppression of effector immune response and thus may contribute to tumorigenesis. Initially it was believed that the protein forkhead box P3 (FOXP3) to be exclusive of these cells, however some studies have found expression of this transcription factor also in epithelial cells, including breast, which may act as a tumor suppressor gene. It is known that genetic polymorphisms may modify FOXP3 functionally or quantitatively. On the other hand, studies have shown that epigenetic regulation, such as DNA methylation is crucial to the expression control of *FOXP3* locus. This study aimed to analyze the allelic variants of genetic polymorphisms g.10403A>G (rs2232365) and g.8048A>C (rs3761548) among breast cancer patients and mammary neoplasia free women, the methylation profile in promoter gene and the genic expression. The AA genotype of g.10403A>G was associated with higher risk of breast cancer development (p=0.046). While GG genotype was correlated with higher Ki-67 (p=0.019) in HER-2 enriched subtype (HER2+) and advanced TNM stages in triple-negative (TN) (p=0.032). The AA genotype of g.8048A>C was correlated with higher Ki-67 (p=0.018) and lower histological grade in HER2+ (p=0.026). GA haplotype was correlated with lower histological grade (p=0.009) and higher Ki-67 (p=0.036) in HER2+ and higher staging in TN (p=0.044), while the AC haplotype was correlated with lower Ki-67 (p=0.005) and staging (p=0.027), in HER2+ and TN, respectively. Furthermore, the AC haplotype was correlated with higher *FOXP3* mRNA expression (p=0.039). The Luminal B subtype with HER2 overexpression (LB) showed 2.6 folds lower FOXP3 mRNA expression in relation to control, while TN and HER2+ showed a slightly higher expression (1.6 and 1.8 folds, respectively). The FOXP3 mRNA expression was not correlated with prognostic parameters. No significant difference in methylation profile was observed between benign, ductal carcinoma in situ and invasive (p=0.96), as well no difference was found between the tumor and adjacent normal tissue (p=0.63). The methylation profile was not correlated with FOXP3 mRNA and protein expression, as well neither with prognostic parameters. A significant correlation was found between FOXP3 protein expression in cytoplasm of breast tumor cells with higher lymph nodes commitment (p=0.01) and a tendency between the FOXP3-positive mononuclear cells with higher histological grade (p=0.068). Our results suggests that in breast tumor the methylation profile is not the only responsible to gene transcriptional regulation, and other mechanisms may be involved, such as genetic polymorphisms, since the AC haplotype showed a correlation with FOXP3 expression. In addition, this haplotype was correlated with lower Ki-67 proliferation cell index and staging, indicating that increased FOXP3 in tumor cell may confer a better prognosis. However when analyzing the protein expression we observed that when located in the cytoplasm of tumor cell the FOXP3 may be correlated to worse prognosis, such as lymph node involvement. Thus, its influence on the prognosis may depend on its location, as well as on the breast tumor subtype.

Key words: Breast cancer. FOXP3. Tregs. genetic polymorphisms. DNA methylation.

BANIN-HIRATA, Bruna Karina. **Análisis de polimorfismos genéticos, expression génica y metilación de la region promotora del factor de transcripción FOXP3: implicaciones en la patogénesis de los subtipos agressivos de cáncer de mama.** 2016. 107p. Tesis Doctoral (Programa de Post grado em Patología Experimental) –Universidad Estadual de Londrina (UEL), Londrina, 2016.

RESUMEN

El grado de heterogeneidad molecular y celular en el cáncer de mama y el gran número de eventos genéticos implicados en el control del crecimiento celular, la diferenciación, la proliferación y la metástasis dependen de la interacción tumor-huésped. El cáncer de mama es un microambiente que incluye, además de las células neoplásicas, las células de estroma circundante y del sistema inmune. Las células T reguladoras (Treg) son importantes en la supresión de la respuesta inmune efectora y por lo tanto pueden contribuir a la tumorigénesis. Inicialmente se creía que la proteína forkhead box 3 (FOXP3) era exclusivo de estas células, sin embargo, algunos estudios han encontrado expresión de este factor de transcripción también en las células epiteliales, incluyendo cáncer de mama, que pueden actuar como un gen supresor de tumor. Se sabe que los polimorfismos pueden modificar el FOXP3 cuantitativamente o funcionalmente. Por otra parte, los estudios han demostrado que la regulación epigenética, tales como la metilación del ADN es fundamental para controlar la expresión de *FOXP3* locus. Este estudio tuvo como objetivo analizar las variantes alélicas g.10403A> G (rs2232365) y g.8048A> C (rs3761548) en pacientes con cáncer de mama y las mujeres sin cáncer, el perfil de metilación de la región promotora del gen y su expresión génica. El genotipo AA del polimorfismo g.10403A> G se asoció con una mayor probabilidad de desarrollar cáncer de mama (p=0,046). Mientras que el genotipo GG se correlaciona con un aumento de Ki-67 (p=0,019) en el subtipo HER2-sobreexpresa (HER2+) y com estadificación TNM más avanzada en el triplo-negativo (TN) (p=0,032). El genotipo AA del polimorfismo g.8048A> C se correlacionó con un aumento de Ki-67 (p = 0,018) y redujo grado histológico em HER2+ (p=0,026). El haplotipo GA se correlacionó con grado histológico más bajo (p=0,009) y mayor Ki-67 (p=0,036) en HER2+ y mayor estadificación en TN (p=0,044), mientras que el haplotipo AC se correlacionó con menor Ki-67 (p=0,005) y estadificación (p=0,027) en los subtipos HER2+ y TN, respectivamente. Además, el haplotipo AC se correlacionó con aumento de expresión de RNAm de *FOXP3* (p=0,039). El subtipo Luminal B HER2+ (LB) mostró 2,6 veces menos expresión de RNAm de *FOXP3* en comparación con el control, mientras que en TN y HER2+ la expresión fueron ligeramente más altos (1,6 y 1,8 veces, respectivamente). La expresión del RNAm de *FOXP3* no se correlacionó con los parámetros pronósticos de los pacientes. No hubo diferencias en el perfil de metilación de tumores benignos, carcinoma ductal in situ e invasivos (p=0,96) y no hubo diferencia entre el tumor y el tejido normal adyacente (p=0,63). El perfil de metilación también no se correlaciona con la expresión de RNAm y proteína de FOXP3, así como parámetros pronósticos. Se observó una correlación significativa entre la expresión citoplasmática de FOXP3 en las células tumorales mamarias con afectación de los ganglios linfáticos (p=0,01) y una tendencia de correlación entre la infiltración de células mononucleares FOXP3-positivas con un grado histológico más alto (p=0,068) . Nuestros resultados sugieren que el perfil de metilación del *FOXP3* em tumor de mama no está relacionado de forma única a la regulación transcripcional de este gen, pueden estar implicados otros mecanismos, como los polimorfismos genéticos, ya que el haplotipo AC de los polimorfismos estudiados mostró una correlación con la expresión del gen . Por otra parte,

este haplotipo se correlacionó con um menor Ki-67 y estadificacióna que indica que FOXP3 aumentado en las células tumorales puede dar un mejor pronóstico. Sin embargo, cuando el análisis de la expresión de proteínas observó que cuando se encuentra en el citoplasma de la célula tumoral FOXP3 puede ser correlacionada con un mal pronóstico y la implicación de los ganglios linfáticos. Por lo tanto su influencia en el pronóstico puede depender de su ubicación, y el subtipo de tumor de mama.

Palabras-clave: Cáncer de mama. FOXP3. Células T reguladoras. Polimorfismo genético. Metilación de ADN.

LISTA DE FIGURAS

LISTA DE TABELAS

LISTA DE ABREVIATURAS E SIGLAS

AP-1	Proteína ativadora 1
ATF2	Fator ativador de transcrição 2
CD	Grupo de diferenciação
CD25	Grupo de diferenciação 25
CDI	Carcinoma Ductal Invasivo
CDIS	Carcinoma Ductal *in situ*
c-Myc	Proto-oncogene Myc (*de avian myelocytomatosis*)
CNS	Sequência não coficadora
CTLA-4	Antígeno 4 associado a linfócito T citotóxico
CXCR4	Receptor 4 para quimiocinas da familia CXC
EGFR	Receptor de fator de crescimento epidérmico
FOXP3	Fator de transcrição *Forkhead box* P*3*
HER2	Receptor tipo 2 de fator de crescimento epidérmico humano
HRP	Peroxidase de raiz forte
IARC	Agência Internacional de Pesquisa em Câncer
IL-17	Interleucina 17
IL-2	Interleucina 2
INCA	Instituto Nacional do Câncer
LB	Luminal B
NFAT	Fator nuclear de células T ativadas
NF-κB	Fator nuclear kappa B
OMS	Organização Mundial da Saúde
p53	Proteína supressora tumoral
PCR	Reação em Cadeia da Polimerase
qRT-PCR	PCR quantitativa em tempo real
RE	Receptor de estrógeno
RNAm	RNA mensageiro
RP	Receptor de progesterona
RUNX1	Fator de transcrição1 relacionado com o gene *RUNT*
SKP2	Proteína quinase-associada da fase S2
SNP	Polimorfismo de nucleotídeo único
T_H	Linfócito T auxiliar

TN	Triplo-negativo
TNM	Tumor-Nódulo-Metástase
Treg	Célula T reguladora
UICC	União Internacional de Controle ao Câncer

SUMÁRIO

1. INTRODUÇÃO

De acordo com o documento World Cancer Report 2014 da *International Agency for Research* (IARC), da Organização Mundial da Saúde (OMS), o câncer é um problema de saúde pública inquestionável, especialmente entre os países em desenvolvimento onde são esperados mais de 20 milhões de novos casos para 2025. A crescente magnitude desta doença é parcialmente uma consequência da crescente exposição a fatores de risco e do aumento da expectativa de vida (INCA, 2016).

De acordo com o Sistema de Informações sobre Mortalidade (SIM), do Ministério da Saúde, as neoplasias ocuparam a segunda posição entre as principais causas de morte no Brasil, de Janeiro a Junho do ano de 2016, representando 18,2% das mortes, seguida apenas das doenças cardiovasculares, com 29,7% (MINISTÉRIO DA SAÚDE, 2016).

O Instituto Nacional de Câncer José Alencar Gomes da Silva (INCA) estima para o Brasil, em 2016, cerca de 57.960 novos casos de câncer de mama, com um risco estimado de 56,20 casos a cada 100 mil mulheres (INCA, 2016). Sem considerar os tumores de pele não melanoma, esse tipo de câncer é o mais frequente na população feminina das Regiões Sul, Sudeste, Centro-Oeste e Nordeste. Na região Norte, é o segundo tumor mais incidente, seguido do câncer de colo de útero.

Dentre os diferentes tipos de câncer, o de mama é a maior causa de morte por câncer nas mulheres em todo o mundo, com aproximadamente 522 mil mortes estimadas para o ano de 2012. O câncer de mama é a segunda causa de morte por câncer nos países desenvolvidos (atrás do câncer de pulmão) e a maior causa de morte por câncer nos países em desenvolvimento. Apesar de ser considerado um tumor relativamente de bom prognóstico, se diagnosticado e tratado oportunamente, as taxas de mortalidade por esta doença continuam elevadas no Brasil (14 óbitos a cada 100 mil mulheres em 2013) (INCA, 2016).

Apesar da alta taxa de mortalidade, a sobrevida em cinco anos está aumentando na maioria dos países desenvolvidos, aproximadamente 85% durante o período de 2005 a 2009. Na América do Sul, particularmente no Brasil, a sobrevida em cinco anos aumentou entre os períodos de 1995 a 1999 e 2005 a 2009 (de 78% para 87%) (INCA, 2016).

A maioria das mortes destes pacientes é ocasionada, principalmente, pela propagação das células tumorais, uma vez que esta neoplasia é caracterizada por um padrão metastático distinto envolvendo linfonodos regionais, medula óssea, pulmão e fígado (REDIG e MCALLISTER, 2013).

O curso clínico e a sobrevida variam para cada paciente e dependem de uma série complexa de fatores. Os fatores de risco incluem a idade, paridade, idade da primeira gestação, amamentação, idade da menarca e menopausa, tratamento com estrógeno, estresse, condição imunológica e nutrição. O histórico familial é outro importante fator de risco, enfatizando o aspecto hereditário desta doença (HONDERMARCK *et al.*, 2001).

O câncer caracteriza-se pela proliferação desregulada de células e surge a partir de alterações essenciais na fisiologia celular, as quais, coletivamente, contribuem para o crescimento dos tumores malignos. Dentre as alterações essenciais podem ser citadas: suficiência em relação aos fatores de crescimento, insensibilidade aos inibidores de crescimento, evasão à morte celular programada, potencial ilimitado de replicação, angiogênese aumentada, indução de processo inflamatório, desregulação do metabolismo energético, evasão do sistema imunológico, instabilidade genômica, invasão tecidual e disseminação à distância (metástase) (HANAHAN e WEINBERG, 2011). A etiologia desta doença é genética, no entanto não é necessariamente hereditária. Os cânceres humanos são, na sua maioria, originados de mutações somáticas resultantes da interação de fatores genéticos e ambientais (PERERA, 1997).

Embora a célula tumoral represente o principal foco no desenvolvimento de uma neoplasia, é importante considerar que a massa tumoral não é composta apenas de células neoplásicas, mas de um conjunto de células tumorais e elementos não neoplásicos, tais como células mesenquimais e componentes dos sistemas vascular e imunológico, que contribuem substancialmente para a carcinogênese, progressão tumoral e metástase das células transformadas (KERKAR e RESTIFO, 2012).

Particularmente, no câncer de mama, evidências crescentes sugerem que as células do sistema imune exercem grande influência sobre o desenvolvimento e a progressão do tumor (GU-TRANTIEN *et al.*, 2013). Assim, a melhor compreensão do papel das células imunológicas, especialmente as células T no microambiente tumoral é essencial para o desenvolvimento de novas estratégias terapêuticas para esta doença.

Está bem estabelecido que uma resposta imune anti-tumoral efetiva requer o envolvimento tanto de células T $CD4^+$ quanto $CD8^+$ (SCHREIBER *et al.*, 2011; HUANG *et al.*, 2015), ambas as populações celulares estão dinamicamente envolvidas na resposta imune do câncer de mama. Nos estágios iniciais da doença há um predomínio de células T $CD4^+$ de fenótipo T_H1 e células T $CD8^+$, que realizam a imunovigilância. No entanto, nos estágios mais tardios da doença há um aumento considerável de células T $CD4^+$ com fenótipos T_H17 e de células T reguladoras (Tregs), as quais contribuem para a progressão do tumor.

Tem sido demonstrado que células Tregs que infiltram o tumor induzem imunossupressão no microambiente tumoral, impedindo uma imunidade anti-tumoral efetiva, tornando-se um obstáculo para a imunoterapia contra o câncer de mama (CURIEL, 2008). As células Tregs apresentam um fator de transcrição denominado proteína *forkhead box* 3 (FOXP3), uma molécula intracelular chave para o seu desenvolvimento e função, sendo considerado o marcador mais específico para estas células (HORI *et al.*, 2003).

Atualmente sabe-se que este fator não é restrito às células Tregs, sendo detectado inclusive em diferentes linhagens de células tumorais, tais como pulmão, cólon, mama, melanoma e leucemias (KARANIKAS *et al.*, 2008). No entanto a significância e as funções biológicas do FOXP3 nas células tumorais não estão bem esclarecidas.

O valor prognóstico do FOXP3 no câncer de mama também permanece contraditório. De acordo com LADOIRE *et al.* (2011) a influência do FOXP3 parece depender do subtipo molecular do câncer de mama, podendo ser um marcador de bom prognóstico em tumores com superexpressão do receptor tipo 2 de fator de crescimento epidérmico humano (HER2) e de prognóstico desfavorável em outros subtipos tumorais. MERLO *et al.* (2009) sugerem que a expressão deste fator de transcrição em células tumorais de mama confere pior prognóstico. Em contraste com estes dados, tem sido demonstrado que o FOXP3 age como um gene supressor tumoral de oncogenes importantes no câncer de mama, tais como *HER2*, proteína quinase associada da fase S2 (*SKP2*) e o proto-oncogene Myc (*c-Myc*) (ZUO, LIU, *et al.*, 2007; ZUO, WANG, *et al.*, 2007; DOUGLASS *et al.*, 2014). As evidências encontradas nos diferentes estudos de que o FOXP3 influencia a progressão tumoral ressalta a importância de mais estudos acerca deste fator de transcrição a fim de esclarecer seu real papel no microambiente tumoral mamário a fim de se estabelecer um possível marcador prognóstico ou mesmo uma nova estratégia terapêutica.

1.1. Classificação clínica e molecular do câncer de mama

O câncer de mama foi tradicionalmente reconhecido como uma doença única, no entanto, os avanços na ciência tem revelado que este carcinoma trata-se, na verdade, de um conjunto de doenças que exibem diferentes características. Esta heterogeneidade representa um desafio para o diagnóstico e o tratamento dos pacientes (TAHERIAN-FARD *et al.*, 2015).

Os tumores mamários são classificados histologicamente de acordo com o local de origem, dividindo-se em ductais e lobulares. Os tumores ductais se desenvolvem dos

ductos mamários e representam cerca de 80% dos tumores. Os tumores lobulares se desenvolvem nos lóbulos e representam de 10 a 15% dos casos. Outros subtipos representam menos de 10% dos casos diagnosticados por ano (VARGO-GOGOLA e ROSEN, 2007). Pacientes com carcinoma ductal invasivo apresentam maior comprometimento linfático e pior prognóstico do que outros tipos de carcinomas mamários (KETTERHAGEN *et al.*, 1984).

O estadiamento do tumor e o grau de diferenciação histológica são classificações bastante utilizadas na clínica e são importantes na orientação do tratamento. O sistema de estadiamento mais utilizado é o Sistema Tumor-Nódulo-Metástase (TNM) de classificação dos Tumores Malignos, preconizado pela União Internacional de Controle ao Câncer (UICC), o qual se baseia na extensão anatômica da doença, considerando as características do tumor primário, nos linfonodos das cadeias de drenagem linfática do órgão em que o tumor se localiza, e na presença ou ausência de metástases. A avaliação desses parâmetros permite a determinação do estadiamento que varia dos estágios I ao IV (SOBIN *et al.*, 2009).

O grau de diferenciação histológica pode ser classificado em I, II ou III, no qual o I indica maior diferenciação histológica e, portanto, apresenta melhor prognóstico por assemelhar-se ao tecido de mama normal, enquanto o grau III corresponde ao tecido menos diferenciado e assemelha-se às células-tronco, indicando, portanto, pior prognóstico para a paciente. Essas classificações são baseadas nos níveis de pleomorfismos nucleares, formação glandular/tubular e índice mitótico (LESTER *et al.*, 2009).

Além da classificação histológica, o carcinoma mamário recebeu uma classificação molecular por Perou e colaboradores em 2000. Esta classificação divide o câncer de mama em quatro subtipos moleculares: luminal, HER2+, basalóide e normal-like (PEROU *et al.*, 2000). No ano seguinte, este mesmo grupo de pesquisa dividiu o subgrupo luminal em dois grupos, luminal A e luminal B, cada um com um perfil de expressão e prognóstico diferentes (SORLIE *et al.*, 2001).

A técnica considerada padrão-ouro para classificação do câncer de mama em subtipos é a de *microarray*, uma ferramenta de análise de expressão gênica que permite investigar a expressão de centenas ou milhares de genes em uma amostra através de uma reação de hibridização (LEONG e ZHUANG, 2011).

No entanto, devido ao elevado custo desta técnica, na prática clínica o carcinoma mamário é classificado através da técnica de imunohistoquímica de acordo com um painel de biomarcadores, tais como receptor de estrógeno (RE), receptor de progesterona (RP), HER2 e índice de proliferação celular Ki-67. Os receptores de estrógeno e progesterona

são receptores hormonais expressos tanto no epitélio quanto no estroma que se ligam a hormônios circulantes mediando seus efeitos celulares (ROSEN, 1987; HASLAM, 1989). O HER2 consiste em um receptor transmembrana tirosina quinase que pertence à família dos receptores de fator de crescimento epidermal, cuja homodimerização ou heterodimerização ativa uma via de sinalização celular que resulta na sobrevivência e proliferação celular (YAMAMOTO *et al.*, 1986; CITRI *et al.*, 2003). Ki-67 consiste em uma proteína nuclear que está estreitamente ligada ao ciclo celular e é expressa em fases de proliferação celular, mas não em células em repouso (GERDES *et al.*, 1984). Juntos, estes quatro marcadores formam a base da definição imunohistoquímica dos subtipos moleculares que foram recomendados pelo consenso de St Gallen de 2011 (GOLDHIRSCH *et al.*, 2013).

O subtipo luminal A é definido como RE/RP positivo, HER2 negativo e baixa expressão de Ki-67, enquanto o subtipo luminal B é definido como RE positivo, podendo ou não ter superexpressão de HER2. Diferentemente do subtipo Luminal A, quando o subtipo luminal B apresenta HER2 negativo o Ki-67 é alto (CHEANG *et al.*, 2009). O luminal A é o subtipo mais comum e representa de 50 a 60% dos tumores mamários enquanto o luminal B está entre 10 a 20%. Comparado ao luminal A, o luminal B apresenta um fenótipo mais agressivo, com maior grau histológico e e pior prognóstico (KENNECKE *et al.*, 2010; JOHANSSON *et al.*, 2014).

Quando comparado com tumores negativos para receptores hormonais, o subtipo Luminal apresenta forte resposta clínica ao tratamento hormonal, aparência morfológica mais diferenciada e maior sobrevida, tanto livre de doença, quanto global (HENDERSON e PATEK, 1998; HARVEY *et al.*, 1999).

O subtipo HER2+ é caracterizado pela superexpressão deste receptor e é tipicamente negativo para receptores de estrógeno e progesterona, exibindo alta expressão de genes associados com a progressão do ciclo celular (SORLIE *et al.*, 2001; SORLIE, 2004). Cerca de 15 a 20% dos pacientes com câncer de mama apresentam este subtipo. Do ponto de vista clinico, é caracterizado por pior prognóstico, embora na última década, o tratamento com anticorpo monoclonal anti-HER2, denominado trastuzumab, tenha aumentado consideravelmente a sobrevida não somente nas doenças metastáticas, como também nos estágios iniciais da doença (PICCART-GEBHART *et al.*, 2005; PARKER *et al.*, 2009).

Aproximadamente 10 a 20% dos tumores mamários são do subtipo basalóide. Este termo foi cunhado porque estes tumores expressam genes geralmente presentes em células mioepiteliais mamárias normais, incluindo as citoqueratinas de alto peso molecular CK5 e CK6, p-caderina, caveolinas, nestina, CD44 e receptor do fator de

crescimento epidermal (EGFR) (BOSCH *et al.*, 2010). Ao longo dos anos, o câncer de mama basalóide tornou-se mais comumente conhecido como triplo-negativo (TN) devido a ausência de expressão de receptores hormonais (RE e RP) e superexpressão de HER2 (PRAT *et al.*, 2013). No entanto, marcadores como EGFR e as citoqueratinas podem estar ausentes em alguns tumores TN, salientando que, embora todos os basalóides sejam TN, nem todos TN são do tipo basalóides. Caracteristicamente, este subtipo possui um comportamento mais agressivo, predileção por metástase visceral, normalmente apresentam alto grau histológico e tendência em afetar mulheres jovens, favorecendo um pior prognóstico (DAWSON *et al.*, 2009; MAEGAWA e TANG, 2010; PRAT *et al.*, 2013). Terapias personalizadas, como a terapia endócrina e anti-HER2, não são aplicáveis ao câncer de mama TN (KUREBAYASHI, 2009). Apesar deste subtipo ser sensível a antraciclinas e taxanos (MAEGAWA e TANG, 2010), a recidiva precoce é comum (CAROTENUTO *et al.*, 2010).

O normal-like representa cerca de 5 a 10% dos carcinomas mamários e são pouco caracterizados, e tem sido agrupados em fibroadenomas e amostras mamárias normais (PEROU *et al.*, 2000). Este subgrupo não possui expressão de RE, HER2 e RP, portanto também podem ser caracterizados como triplo-negativos, mas não basalóides, uma vez que são negativos para CD5 e EGFR. Existem dúvidas sobre sua real existência e alguns pesquisadores acreditam que podem ser um artefato de técnica de alta contaminação com tecido normal (WEIGELT *et al.*, 2010).

Em 2011, LEHMANN *et al.* (2011) analizaram a expressão gênica em tumores de mama e identificaram seis subtipos TN que mostraram diferentes respostas à terapias. Dentro deste contexto, fica claro que o câncer de mama não é uma doença única, mas um conjunto de doenças, e que cada paciente constitui um caso particular onde a medicina personalizada, selecionada com base em biomarcadores específicos, desempenha um importante papel no tratamento das pacientes com câncer de mama.

1.2. Células T regulatórias e câncer de mama

A patogênese do câncer é iniciada e modulada pela interação entre as células malignas transformadas, o estroma circundante e o sistema imune inato e adaptativo. Essas interações são complexas e componentes do sistema imune atuam tanto na defesa como contribuindo para a iniciação, crescimento, invasividade e desenvolvimento de metástase (YAQUB e AANDAHL, 2009). A interação entre os tumores e seu microambiente é

complexa e difícil de decifrar e sua compreensão é fundamental para o desenvolvimento de novos marcadores prognósticos e estratégias terapêuticas (FRIDMAN *et al.*, 2012).

O papel das células T $CD4^+$ na imunidade antitumoral tem sido extensivamente estudado tanto em modelos animais quanto em pacientes com câncer. As células T $CD4^+$ são essenciais para a ativação e proliferação de células T $CD8^+$ tumor-específicas, bem como geração de células T $CD8^+$ de memória (JANSSEN *et al.*, 2003). Contudo, a descoberta das Tregs e das células T_H17 não somente mudou o clássico paradigma de diferenciação de células T auxiliares T_H1/T_H2, como também alterou marcadamente o pensamento tradicional sobre o papel que as células $CD4^+$ desempenham na resposta imune tumoral (WANG *et al.*, 2004; YE *et al.*, 2013).

Nos estágios iniciais do câncer de mama parece haver um predomínio de células T $CD8^+$ e auxiliares com fenótipo T_H1 que desempenham imunidade antitumoral, enquanto nos estágios mais tardios da doença há uma mudança de células T $CD4^+$ de fenótipo T_H1 para Tregs e T_H17 que estão mais relacionadas à progressão tumoral e pior prognóstico da doença (HUANG *et al.*, 2015).

Sob condições normais, as Tregs constituem uma linhagem de células T $CD4^+$ que desempenham um papel indispensável na tolerância imunológica aos auto-antígenos e na supressão de respostas imunes excessivas, consideradas deletérias ao hospedeiro. No entanto, muitos estudos têm demonstrado que estas células também podem inibir a resposta imune efetora antitumoral e desta forma contribuir para o desenvolvimento do câncer (SAKAGUCHI *et al.*, 2008; VIGNALI *et al.*, 2008; TOKER e HUEHN, 2011).

Em uma meta-análise realizada por SHANG *et al.* (2015), o elevado infiltrado de células Tregs foi significativamente associado com pior prognóstico na maioria dos tumores sólidos, incluindo o câncer de mama, no entanto em carcinomas como o de cabeça e pescoço, de esôfago e colorretal, foi associado com prognóstico favorável. Isso indica que o valor prognóstico do infiltrado de células T reguladoras no câncer permanece controverso, podendo ser altamente influenciado pela localização do tumor, subtipo molecular e estágio tumoral.

Além disso, isto mostra que a visão de que as células Tregs apenas suprimem a imunidade tumoral é simplificada. Os efeitos prognósticos do infiltrado de Tregs observados nos diferentes cânceres, pode ser devido à diferentes propriedades biológicas específicas dos tipos tumorais, e o impacto positivo das Tregs pode estar relacionado ao seu efeito anti-inflamatório em muitos tumores (SHANG *et al.*, 2015).

O valor prognóstico do infiltrado de Tregs em tumor mamário varia bastante entre diferentes estudos. Estes resultados controversos podem ser explicados em parte devido aos diferentes subtipos de câncer de mama. SHANG *et al.* (2015) realizaram uma análise estratificando os tumores de acordo com o *status* de receptor de estrógeno, e encontraram alto número de Tregs associado com resultados clínicos favoráveis em tumores negativos para RE, enquanto em tumores RE-positivos o infiltrado foi associado com prognóstico desfavorável. HUANG *et al.* (2015) encontraram correlação positiva entre o infiltrado de células Tregs no tumor mamário com estágios tumorais mais avançados, maior tamanho de tumor, comprometimento de linfonodos e expressão de HER2 e correlação negativa com sobrevida livre de recorrência.

1.3. *Forkhead Box* P*3* (FOXP3)

A proteína forkhead box P3 (FOXP3) é um fator de transcrição, cuja função é exercida sobre regiões reguladoras específicas dentro do DNA, sendo essencial para o desenvolvimento e função de Tregs (FONTENOT *et al.*, 2003; HORI *et al.*, 2003). Foi identificado no ano de 2001 como o causador de uma doença em murinos *scurfy*, que desenvolvem autoimunidade grave espontaneamente e/ou inflamação, como resultado de uma mutação de base única no cromossomo X (BRUNKOW *et al.*, 2001).

FOXP3 contém uma região amino terminal denominada domínio repressor, necessária para regulação transcricional, um dedo de zinco central, ainda de função desconhecida, um zíper de leucina implicado na formação de oligômero e um domínio altamente conservado denominado *forkhead* (FKH) na região carboxi-terminal, responsável pela ligação ao DNA (CHEN *et al.*, 2015).

Estima-se que este fator de transcrição possa se ligar a aproximadamente 700 genes, desempenhando tanto atividade transcricional de ativação quanto de repressão (ZHENG *et al.*, 2007). Os mecanismos moleculares pelos quais o FOXP3 medeia a regulação transcricional não estão bem estabelecidos, mas existem algumas evidências de que ele se liga ao DNA complexado a outros fatores de transcrição, tais como RUNX1 (RECOUVREUX *et al.*, 2016), NFAT e NF-κB (BETTELLI *et al.*, 2005).

A capacidade do FOXP3 de agir tanto como repressor quanto ativador transcricional é mais bem demonstrada nas células Tregs, nas quais ele promove a transcrição de CTLA-4 e CD25 e reprime a transcrição de IL-2 e IL-17, características das Tregs e por isso é considerado um marcador chave para estas células (DOUGLASS *et al.*, 2012).

Curiosamente, muitos estudos tem encontrado a expressão de FOXP3, também em células epiteliais de diferentes tipos de câncer, tais como próstata (WANG *et al.*, 2009), ovário (ZHANG e SUN, 2010), melanoma (QUAGLINO *et al.*, 2011), esôfago (XUE *et al.*, 2010) e mama (LADOIRE *et al.*, 2011; LOPES *et al.*, 2014), levantando questões sobre seu papel na carcinogênese.

Embora sua função nas células tumorais não esteja bem esclarecida, alguns estudos têm demonstrado que o *FOXP3* pode atuar como gene supressor tumoral. HAO *et al.* (2014) observaram que no câncer gástrico, este fator de transcrição é capaz de inibir o NF-κB, o qual está estritamente correlacionado com a expressão da ciclooxigenase-2, uma molécula chave no desenvolvimento da doença. LI *et al.* (2011) também encontraram que o FOXP3 é necessário para a expressão de LATS2, um regulador negativo da oncoproteína YAP, relatada em cânceres de próstata, mama, fígado e cérebro. No câncer de próstata e em glioma, FOXP3 tem sido descrito como um repressor transcricional do oncogene *c-MYC* (WANG *et al.*, 2009; FRATTINI *et al.*, 2012).

Particularmente no câncer de mama o papel de FOXP3 ainda é controverso. Tem sido demonstrado que este fator de transcrição pode atuar como um gene supressor tumoral, inibindo oncogenes importantes como *HER2*, *SKP2* e *c-Myc*, assim como o gene do receptor de quimiocinas *CXCR4*, envolvido no processo metástatico do tumor mamário, desta forma conferindo melhor prognóstico (DOUGLASS *et al.*, 2014; ZHANG *et al.*, 2015). Um estudo desenvolvido por RECOUVREUX *et al.* (2016) mostrou ainda que o FOXP3 pode interagir com RUNX1, bloqueando sua atividade, e consequentemente inibindo a expressão do oncogene *RSPO3* e promovendo a do gene supressor tumoral *GJA1*. Além disso, FOXP3 também induz a expressão dos microRNAs miR-146a/b, miR-7 e miR-155, os quais contribuem para a inibição do crescimento tumoral de mama (MCINNES *et al.*, 2012; LIU *et al.*, 2015).

Por outro lado, a expressão proteica de FOXP3, predominantemente citoplasmática, também tem sido associada com pior sobrevida geral, maior Ki-67, maior grau nuclear e histológico, sugerindo também um pior prognóstico (WOLF *et al.*, 2007; MERLO *et al.*, 2009; KIM *et al.*, 2013; WON *et al.*, 2013). Assim sendo, apesar de seu potencial como marcador prognóstico, outros estudos são necessários para esclarecer seu papel na progressão tumoral da neoplasia mamária.

1.3.1. Alterações genéticas e epigenéticas no gene *FOXP3*

O gene *FOXP3* humano está localizado no braço curto do cromossomo X na posição 11.23, contem 11 éxons codificantes e três não-codificantes (-2a, -2b e -1) (Figura 1) (BENNETT *et al.*, 2001; ZIEGLER, 2006). São encontradas em células humanas três variantes de *FOXP3* geradas por *splicing* alternativo, conferindo maior complexidade a este fator de transcrição. A primeira isoforma corresponde à proteína completa com 431 aminoácidos e peso molecular de 47,3 kDa. A segunda isoforma não possui o éxon 2 (FOXP3ΔEx2), que codifica parte do domínio repressor e a terceira não possui o éxon 7 (FOXP3ΔEx7), que codifica parte do zíper de leucina (SMITH *et al.*, 2006).

Tem sido descritos polimorfismos de nucleotídeo único (SNPs) em várias regiões do gene *FOXP3*, tais como região promotora, intrônica ou não codificante e exônica ou codificante (ODA *et al.*, 2013). Polimorfismos podem modificar o FOXP3 funcionalmente ou quantitativamente, e trabalhos do tipo caso-controle envolvendo estes polimorfismos, principalmente em doenças autoimunes e cânceres têm sido desenvolvidos (GAO *et al.*, 2010; ODA *et al.*, 2013).

Dentre os diferentes polimorfismos genéticos descritos em regiões reguladoras do gene, os polimorfismos g.10403A>G (rs2232365) e g.8048A>C (rs3761548) estão localizados no intron-1 do gene *FOXP3* (Figura 1) e tem sido associados com doenças auto-imunes, tais como vitiligo (JAHAN *et al.*, 2013; SONG *et al.*, 2013) e psoríase (SHEN *et al.*, 2010; SONG *et al.*, 2012), evidenciando sua importância no funcionamento de células Tregs. Além disso, o polimorfismo g.8048A>C também tem sido associado com diferentes tipos de cânceres, tais como, colorretal (TANG *et al.*, 2014), pulmão, hepatocelular (JIANG e RUAN, 2014) e mama (LOPES *et al.*, 2014). No entanto, até o momento, não foram encontrados trabalhos de associação do polimorfismo g.10403A>G em qualquer tipo de câncer.

De acordo com um trabalho desenvolvido por SHEN *et al.* (2010), a troca do alelo C para o A no polimorfismo g.8048A>C causa a perda do sítio de ligação de fatores de transcrição, como E47 e c-Myb, diminuindo a transcrição do gene *FOXP3*. Já o segundo polimorfismo parece consistir em um sítio de ligação para o fator de transcrição GATA-3, o qual se liga somente na presença do alelo A (WU *et al.*, 2012). Um estudo desenvolvido por WANG *et al.* (2011) sugere que a combinação de GATA-3 e do próprio FOXP3 são indispensáveis para a expressão de FOXP3. Dessa forma, o polimorfismo g.10403A>G também pode ter um papel na regulação transcricional desse gene.

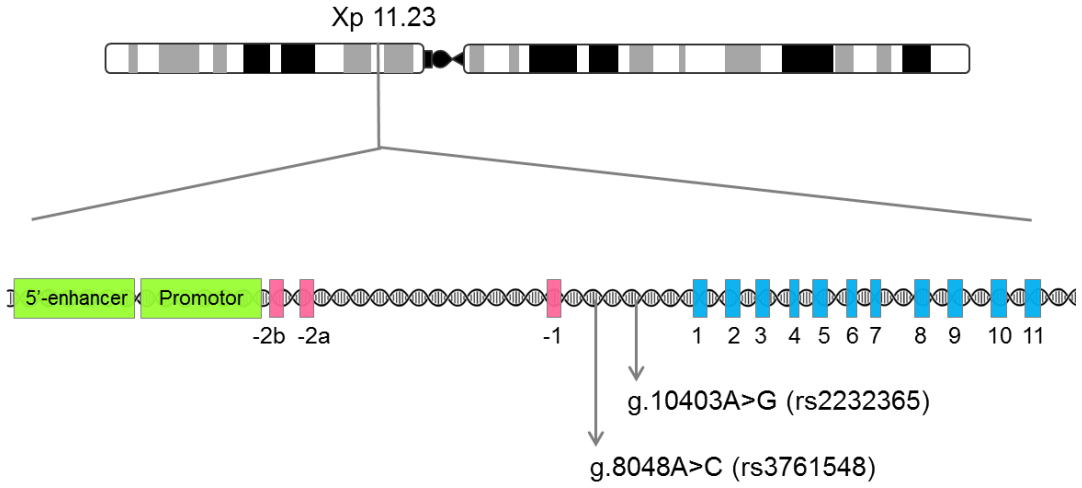

Figura 1. Estrutura esquemática do gene *FOXP3* e localização dos polimorfismos genéticos g.10403A>G (rs2232365) e g.8048A>C (rs3761548). Caixas coloridas de rosa representam éxons não codificantes e caixas coloridas de azul representam éxons codificantes.

Fonte: próprio autor.

No entanto, apesar de estarem associados à expressão de FOXP3, os polimorfismos genéticos correspondem apenas a uma fração do mecanismo de controle transcricional gênico. As regiões não codificantes altamente conservadas do lócus *FOXP3* parecem estar sujeitas a modificações epigenéticas, as quais fornecem uma regulação adicional da transcrição gênica (HUEHN *et al.*, 2009).

Alterações epigenéticas são definidas como modificações do genoma que são herdadas, mas que não alteram a sequência do DNA. São importantes na regulação da expressão gênica e fazem parte do funcionamento celular normal. Metilação de DNA, modificações de histona e RNA não codificadores são três mecanismos epigenéticos, dos quais o primeiro é considerado o principal em mamíferos e é conhecido por ter efeitos potenciais sobre a expressão gênica (SAHIN *et al.*, 2010)

A metilação de DNA é um processo catalisado por DNA metiltransferases (DNMT) que adicionam um grupamento metil na posição C5 de citosinas presentes em ilhas CpG de numerosas regiões promotoras. Desta forma, as DNMTs geram 5-metilcitosina e causam hipermetilação do DNA. Esta modificação interfere na ligação de fatores de transcrição, resultando na repressão da expressão gênica e consequentemente no silenciamento gênico (EHRLICH *et al.*, 1982). Esta alteração epigenética pode representar um passo inicial e fundamental através do qual o tecido normal sofre transformação neoplásica (WAJED *et al.*, 2001).

A primeira região altamente conservada do gene *FOXP3* é a promotora, que está localizado 6,5 kb a montante do primeiro éxon, ativado através da ligação de NFAT e AP1 (MANTEL *et al.*, 2006). A segunda região foi identificada como um elemento sensível à TGFβ, que contém sítios de ligação para NFAT e SMADs (TONE *et al.*, 2008). As diferenças marcantes em relação ao padrão de metilação no lócus *FOXP3* têm sido observadas em uma região altamente conservada rica em CpG, referida como "região demetilada específica de células Treg" (TSDR), por ser totalmente demetilada em células Treg e metilada em células T convencionais (FLOESS *et al.*, 2007; KIM e LEONARD, 2007; NAGAR *et al.*, 2008). O controle epigenético do gene *FOXP3* tem sido amplamente estudado em células T reguladoras (HUEHN *et al.*, 2009), mas não está bem estabelecido em células epiteliais, como as tumorais.

Padrões de metilação de DNA alterados são característicos nos cânceres humanos. Regiões promotoras que normalmente são hipometiladas podem se tornar densamente metiladas e resultar no silenciamento de genes críticos, como os supressores tumorais (JONES e BAYLIN, 2007).

Foram relatados mais de 100 genes hipermetilados em tumores de mama ou em linhagens de câncer de mama (HINSHELWOOD e CLARK, 2008). Muitos desses desempenham papéis importantes na regulação do ciclo celular, apoptose, invasão tecidual e metástase, angiogênese e sinalização hormonal (WIDSCHWENDTER e JONES, 2002).

Sabe-se que o *FOXP3* é um potencial candidato a gene supressor tumoral no câncer de mama, por outro lado quando expresso em células T regulatórias pode estar relacionado à evasão tumoral do sistema imunológico. Dentro deste contexto a avaliação do perfil de metilação e polimorfismos genéticos no gene *FOXP3*, bem como a análise da expressão gênica podem fornecer informações relevantes sobre a biologia do tumor, bem como o impacto de FOXP3 sobre o prognóstico da doença, particularmente em subtipos específicos do carcinoma mamário.

2. OBJETIVOS

Objetivo geral

- Analisar modificações genéticas e epigenéticas no fator de transcrição *FOXP3* e suas possíveis implicações na patogênese do câncer de mama, com foco nos subtipos mais agressivos da doença, Luminal B HER2-positivo, HER2-superexpresso e triplo-negativo.

Objetivos específicos

- Desenvolver um trabalho de revisão bibliográfica com foco no valor prognóstico do infiltrado de células T regulatórias em tumor mamário, com ênfase no subtipo HER2+.

- Analisar os genótipos e haplótipos dos polimorfismos g.10403A>G (rs2232365) e g.8048A>C (rs37861548) do gene *FOXP3* em pacientes com subtipos agressivos de câncer de mama e em mulheres livres de neoplasia, num estudo de associação do tipo caso-controle;

- Analisar a expressão gênica de *FOXP3* em subtipos agressivos do câncer de mama, e correlacionar com polimorfismos genéticos e com os parâmetros prognósticos das pacientes;

- Quantificar a porcentagem de metilação da região promotora do gene *FOXP3* em tecidos normais e tumorais de mama, e correlacionar com sua expressão gênica e proteica, bem como com os parâmetros prognósticos das pacientes.

3. MATERIAIS E MÉTODOS

3.1. Aspectos éticos

Este projeto foi aprovado pelo Comitê de Ética em Pesquisa Envolvendo Seres Humanos – Universidade Estadual de Londrina (CEP/UEL No. 189/2013), o qual está de acordo com a Comissão Nacional de Ética em Pesquisa (CAAE - 171231134000005231) (Anexo A). Todas as participantes assinaram o Termo de Consentimento Livre e Esclarecido (Anexo B).

3.2. Seleção e caracterização da amostra

As amostras utilizadas neste estudo incluíram: sangue periférico (5 mL), coletado com anticoagulante EDTA e tecido tumoral incluído em parafina, para análises de polimorfismos genéticos, e tecidos tumoral e normal adjacente à fresco para análise do perfil gênico de metilação e expressão de RNAm de FOXP3. Os tecidos incluídos em parafina também foram utilizados para análise imunohistoquímica.

Para o estudo de polimorfismos genéticos foram obtidas 117 amostras de pacientes com câncer de mama atendidas no Hospital do Câncer de Londrina (HCL), Londrina, Paraná, Brasil. Destas amostras, 37 foram diagnosticadas como Luminal B HER2+ (LB), 26 como HER2-superexpressas (HER2+) e 54 como triplo-negativas. Do total, foi analisada a expressão gênica de *FOXP3* em 61 amostras.

Para o grupo controle, foram coletadas 300 amostras de sangue periférico de mulheres sem câncer de mama, comprovado por exame clínico e de imagem e sem histórico familial de câncer. Estas amostras foram coletadas de mulheres atendidas na Unidade Básica de Saúde (UBS) Orlando Sestari e Ambulatório de Especialidades do Hospital Universitário (AEHU), Londrina, Paraná. Adicionalmente, para análise do perfil de metilação da região promotora de *FOXP3* foram obtidas 25 amostras de tecido à fresco de tumor e de tecido normal adjacente, das quais duas foram diagnosticadas como carcinoma *in situ* (CIS), 20 como carcinoma ductal invasivo (CDI) e três como tumores benignos (BG).

Os dados clinicopatológicos das pacientes foram gentilmente fornecidos pelo Sistema de Arquivo Médico e Estatística (SAME) do HCL. Os parâmetros incluíram: tamanho tumoral, comprometimento de linfonodos, índice de proliferação celular Ki-67, grau histológico, *status* de RP, RE e HER2 e estadiamento TNM (Tumor-Nódulo-Metástase), o qual foi determinado de acordo com o critério de classificação estabelecido pela União Internacional para o Controle do Câncer (SOBIN *et al.*, 2009).

3.3. Extração de DNA genômico

O DNA genômico de sangue periférico foi obtido através do kit Biopur Mini Spin Plus (Biometrix Diagnostica, Curitiba, Brasil), seguindo as instruções do fabricante. Para extração de DNA a partir de tecidos incluídos em blocos de parafina, foi utilizado o kit innuPREP DNA Mini (Analytik Jena, Jena, Germany). Para a extração de DNA dos tecidos à fresco foi utilizado o kit QIAamp DNA Mini Kit (Qiagen Company, Hilden, Germany). As amostras de DNA foram ressuspendidas em 50ul de tampão de eluição, quantificadas no aparelho NanoDrop 2000c®Spectrophotometer (Thermo Scientific, Wilmington, Estados Unidos da América [EUA]) e armazenadas até o momento de uso a -20^{o}C.

3.4. Extração de RNA do tecido mamário à fresco

As células de tecido mamário tumoral foram homogeneizadas em TRIzol-LS (Invitrogen, São Paulo, Brasil) de acordo com as instruções do fabricante. O RNA obtido foi ressuspendido em água tratada com DietilPirocarbonato (DEPC; LGC Biotecnologia, São Paulo, Brasil). As amostras foram quantificadas no NanoDrop 2000c®Spectrophotometer (Thermo Scientific, Wilmington, EUA). Apenas as amostras que apresentaram relações de absorbâncias próximas de 1,9 (260/280) foram utilizadas nos protocolos de análise de expressão gênica. Antes dos ensaios com RNAm de *FOXP3*, foi realizada a validação das amostras através de PCR convencional para o gene de *β-actina* utilizando primers específicos, conforme descrito por AMARANTE *et al.* (2005). O fragmento amplificado de 353 pares de bases foi analisado por eletroforese em gel de poliacrilamida a 10% corado com Nitrato de Prata ($AgNO_3$).

3.5. Análise dos polimorfismos genéticos de *FOXP3*

Aproximadamente 100 ng de DNA foram amplificados com *primers* específicos para os dois polimorfismos propostos g.10403A>G (rs2232365) e g.8048A>C (rs3761548), sintetizados de acordo com PARADOWSKA-GORYCKA *et al.* (2015) e HE *et al.* (2013), respectivamente (Tabela 1).

As reações de amplificação foram realizadas em um volume final de 25µl com 0,1mM de dNTP, 0,2µM de cada iniciador,0,8mM de $MgCl_2$, Tampão 1x (20mM de Tris-HCl ph 8,5; 50mM de KCl), albumina sérica bovina 0,8x e 1,25U de Taq polimerase

(Invitrogen, Carlsbad, EUA). Para todas as reações foram realizados controles negativos para certificar a ausência de contaminação.

Os ciclos de PCR foram iguais para os dois polimorfismos, com exceção da temperatura de anelamento que foi de 59ºC para o polimorfismo g.10403A>G e de 65ºC para o polimorfismo g.8048A>C. O ciclo foi constituído de uma etapa de desnaturação de 5 minutos a 94^{o}C, seguida de 35 ciclos de 94ºC por 45 segundos, 59ºC ou 65ºC por 45 segundos e 72ºC por 45 segundos, com extensão final de 10 minutos a 72ºC.

Os produtos de PCR (5µl) de g.10403A>G foram submetidos a digestão enzimática durante 12 horas a 55ºC com 1 unidade/reação de enzima de restrição *BsmBI* (New England Biolabs, Beverly, EUA), e os produtos de PCR (6µl) de g.8048A>C foram digeridos durante 12 horas a 37ºC com 2 unidades/reação de enzima de restrição *PstI* (New England Biolabs, Beverly, EUA). Os produtos foram analisados em gel de poliacrilamida (10%), corados com nitrato de prata. Iniciadores específicos, produtos de PCR e os fragmentos de restrição estão apresentados na Tabela 1.

Tabela 1. Sequência de oligonucleotídeos, enzimas de restrição, e tamanhos dos fragmentos do produto de PCR e dos produtos de clivagem dos polimorfismos genéticos g.10403A>G (rs2232365) e g.8048A>C (rs3761548).

	Iniciadores	Produto PCR	Enzima de restrição	Produtos de restrição
g.10403A>G	F: 5' - AGGAGAAGGAGTGGGCATTT – 3' R: 5' - TGTGAGTGGAGGAGCTGAGG – 3'	249pb	BsmBI	Alelo A: 249pb Alelo G: 132, 117pb
g.8048A>C	F: 5' - GGCAGAGTTGAAATCCAAGC – 3' R: 5' - CAACGTGTGAGAAGGCAGAA – 3'	155pb	PstI	Alelo A: 155pb Alelo C: 80, 75pb

F: *primer Forward*; R: *primer Reverse*

3.6. Análise de haplótipos

Os haplótipos dos polimorfismos g.10403A>G (rs2232365) e g.8048A>C (rs3761548) do gene *FOXP3* foram determinados com base nos genótipos de todos os participantes do estudo usando o *software* PHASE versão 2.1.1 (STEPHENS *et al.*, 2001; STEPHENS e SCHEET, 2005). O teste de permutação também foi realizado neste *software* para verificar se há diferenças de distribuição de haplótipos entre pacientes controles e com câncer de mama.

3.7. Reação de Transcrição Reversa (RT-PCR)

A síntese de DNA complementar foi realizado a partir de 500ng de RNA e 20U de transcriptase reversa Moloney Murine Leukemia Virus Reverse Transcriptase M-

MLV RT (Invitrogen, Carlsbad, EUA) e 4U de Inibidor de Ribonuclease Recombinante RNaseOUT (Invitrogen, Carlsbad, EUA), sob as seguintes condições: 0,4μM de oligo dT, 40mM de Tris-HCl (pH 8,3), 60mM de KCl, 6,4 mM de $MgCl_2$ e 0,2 mM de dNTP a 37ºC por 60 minutos.

3.8. PCR Quantitativa em Tempo Real (qRT-PCR)

A PCR quantitativa em tempo real foi realizada utilizando o fluoróforo Platinum SYBR Green qPCR SuperMix UDG (Invitrogen, Carlsbad, EUA) no termociclador Step One Real-Time PCR (Applied Biosystems, Foster City, EUA). Os iniciadores utilizados para amplificação de *FOXP3* e *β-actina* estão descritos na Tabela 2. As reações de PCR foram realizadas em duplicata, conforme as seguintes condições de ciclagem: 10 minutos a 95ºC, 40 ciclos de 15 segundos a 95ºC e 1 minuto a 60ºC, seguido da construção da curva de dissociação através de um ciclo de 15 segundos a 95ºC, 1 minuto a 60ºC e aumento gradual até 95ºC (0,3ºC/segundo) com detecção de fluorescência a cada aumento de temperatura para confirmar a amplificação específica. A análise da curva de *melting* foi realizada ao final da reação para verificar artefatos como dímeros de *primers* e contaminação. Além disso, em todos os experimentos, controles negativos foram empregados para excluir ou detectar qualquer eventual contaminação. A expressão gênica relativa de *FOXP3* foi calculada de acordo com o método $2^{-\Delta\Delta Ct}$ (LIVAK e SCHMITTGEN, 2001) normalizado com o gene *β-actina*. Um *pool* de RNA comercial de glândula mamária humana (Clontech Laboratories, Mountain View, EUA) foi utilizado como controle não neoplásico.

Tabela 2. Sequência de *primers* da PCR quantitativa em tempo real.

Gene	Número de acesso *GenBank*	*Primer*	Sequência	Melting (TºC)
FOXP3	NM_014009.3	*Forward* *Reverse*	5' – CACCTGGCTGGGAAAATGG - 3' 5' - GAGCCCTTGTCGGATGAT - 3'	86ºC
β-actin	NM_001101	*Forward* *Reverse*	5' – GGCTTTATTTGTTTTTTTTGTTTTG - 3' 5' - CACCTTCACCGTTCCAGTTTTT - 3'	73ºC

3.9. Quantificação da porcentagem metilação da região promotora do gene *FOXP3*

Para análise do perfil de metilação do gene *FOXP3*, primeiramente as amostras foram tratadas com enzimas de restrição sensíveis e dependentes de metilação, utilizando o kit EpiTect Methyl II DNA Restriction (Qiagen Company, Hilden, Germany).

Após a digestão o DNA foi quantificado em um termociclador StepOne real-time system (Applied Biosystems, Foster City, EUA) utilizando o kit RT^2 SYBR Green ROX qPCR Mastermix (Qiagen Company, Hilden, Germany) e *primers* específicos para região promotora de *FOXP3*, *Primer Assay for Human FOXP3*, PPP1R3F (CpG Island 27609) (Qiagen Company, Hilden, Germany). A porcentagem de metilação foi calculada através da planilha EpiTect Methyl DNA methylation PCR (Qiagen Company, Hilden, Germany http://www.sabiosciences.com/dna_methylation_data_analysis.php). As amostras foram divididas em três grupos: hipermetilado, se a porcentagem de metilação do DNA foi maior do que a soma da média das amostras normais mais duas vezes o desvio padrão, metilação normal, se a porcentagem de metilação foi entre a média das amostras normais mais duas vezes o desvio padrão, para mais ou para menos e hipometilado se a porcentagem de DNA metilado foi menor que a média das amostras normais menos duas vezes o desvio padrão (KLAJIC *et al.*, 2013).

3.10. Análise imunohistoquímica

Foram obtidos cortes histológicos de 3 μm de tecido a partir de amostras de tumores de mama incluídos em parafina. As amostras foram desparafinadas por aquecimento a 56°C em xileno e reidratadas gradualmente em soluções de álcool. A recuperação antigênica foi realizada com tampão citrato 8,2mM e ácido cítrico 1,8mM. Os cortes foram incubados com anticorpo monoclonal de camundongo específico para FOXP3 clone 236A/E7 (eBioscience, San Diego, Califórnia, USA) em diluição 1:50 *overnight* a 4ºC em câmara úmida. Os cortes foram estabilizados à temperatura ambiente durante 30 min e lavados com PBS (solução salina tamponada com fosfato). Foi aplicado anticorpo secundário anti-camundongo/coelho conjugado a peroxidase de raiz forte (*horseadish peroxidase*/ HRP) (Bio SB Inc. Santa Barbara, CA, EUA), seguido do cromógeno diaminobenzidina (DAB) (Sigma-Aldrich, EUA).

FOXP3 foi avaliado no tecido tumoral e normal adjacente. A leitura foi realizada sob um microscópio óptico (Eclipse-E200, Nikon, Japão) por patologistas especializadas. Foi adotado o sistema de pontuação semiquantitativo, considerando a intensidade de marcação imunohistoquímica e extensão da área, que tem sido amplamente aceito e utilizado em estudos anteriores de KOK *et al.* (2010). Para cada corte foi dada uma pontuação de acordo com a intensidade da marcação: marcação fraca = 1, marcação moderada = 2 e marcação forte = 3.

Foram realizadas marcações controles para verificar a especificidade do anticorpo primário e todas as análises foram feitas independentemente por pelo menos dois patologistas. No entanto, se houvesse discrepância nas pontuações individuais, os cortes histológicos eram reavaliados objetivando concordância.

3.11. Análise Estatística

As análises de associação para o estudo caso-controle foram realizadas pelo cálculo da *Odds Ratio* (OR) controlada pela idade através de regressão logística binária. Para os polimorfismos individuais foram testados os modelos genotípicos (heterozigotos ou homozigotos variantes contra homozigotos selvagens), dominante (heterozigotos e homozigotos variantes contra homozigotos selvagens) e recessivo (homozigotos variantes contra homozigotos selvagens e heterozigotos). Análises de correlação envolvendo polimorfismos genéticos, haplótipos e parâmetros clinicopatológicos foram realizadas pelo teste Tau-b de Kendall. A comparação de expressão gênica relativa de *FOXP3* entre os diferentes subtipos de câncer de mama, e a comparação do perfil de metilação entre os tumores benignos, IS e CDI foram realizadas pelo teste de Kruskal-Wallis. A comparação do perfil de metilação entre o tecido tumoral e normal adjacente foi realizado pelo teste de Wilcoxon. As análises de correlação da expressão gênica relativa de *FOXP3* e da porcentagem de metilação com os parâmetros prognósticos das pacientes foram realizadas pelo teste de Spearman Rho. As análises foram realizadas através dos *softwares* estatísticos IBM SPSS Statistics versão 22.0 (IBM, New York, USA) e GraphPad Prism versão 6.0 (GraphPad Software, La Jolla, CA, USA). Para todas as análises o nível de significância adotado foi de 5%.

4. PRODUÇÃO BIBLIOGRÁFICA

ARTIGO 1

The prognostic value of regulatory T cells infiltration in HER2-enriched breast cancer microenvironment

Bruna K. Banin-Hirata[a], Carlos E. C. de Oliveira[a], Roberta Losi-Guembarovski[a], Patricia M. M. Ozawa[a], Glauco A. F. Vitiello[a], Felipe C. de Almeida[a], Daniele R. Derossi[b], Nayara D. André[c], Maria A. E. Watanabe[a,*]

[a]Laboratory of Study and Application of DNA Polymorphisms and Immunology, Department of Pathological Sciences, Biological Sciences Center, State University of Londrina, Pr 445 Km 380 Celso Garcia Cid highway, Londrina, Paraná, Brazil. Telephone: +554333715629, e-mail: maewatuel@gmail.com
[b]Cancer Hospital of Londrina, Department of Human Pathology, Clinical Analysis and Toxicology, Health Sciences Center, State University of Londrina, Parana, Brazil.
[c] Department of Biochemistry, Federal University of São João del-Rei, Divinópolis, Minas Gerais, Brazil.

Abstract. Breast cancer represents a complex and heterogeneous disease that comprises distinct diseases conditions, histological features and clinical outcome. Since many years ago, it has been demonstrated an association between HER2 amplification and poor prognosis, because its overexpression is associated with an aggressive phenotype of breast tumor cells. A significant proportion of cases have developed resistance to the current therapies available. Consequently, new prognostic markers are urgently needed to identify patients who are at the highest risk for developing metastases. During the past decade, new insights provided valuable knowledge regarding mechanisms underlying the dynamic interplayed between immune cells and tumor progression. It has been shown that the presence of a lymphocytic infiltrate, particularly of regulatory T cells, in cancer tissue, is associated with clinical outcome promoting rather than inhibiting cancer development and progression. It has been also verified that the clinical value of lymphocytic infiltration in breast cancers could be subtype-dependent, including the HER2-enriched subtype. In this context, this work proposed to discuss the prognostic value of regulatory T cell infiltration in microenvironment of HER2-enriched breast cancer.

Keywords: Breast cancer, HER2-overexpression, Tregs, lymphocyte infiltration

Introduction

Breast cancer (BC) is a heterogeneous disease, comprehending a diversity of etiological, histological and clinical characteristics. Determining tumor markers would significantly improve clinical management in cancer patients, assisting in diagnostic, staging, evaluation of therapeutic response, detection of recurrence and metastasis and development of new treatment modalities. Therefore, an intense search for markers that may be crucial in the course of disease, especially those with prognostic and therapeutic purposes, is urgently required to develop a personalized treatment (1).

BC could be classified into different subgroups with distinct biology basing on molecular profiling. The status of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor type 2 (HER2) has been used as predictive markers to identify a high-risk phenotype and for selection of the most efficient therapies (2, 3). Hence, there are some cases with particular specificities which cannot be included in the traditional groups.

HER2-enriched BC represents a peculiar subtype with aggressive clinical characteristics, however, the addition of HER2-targeted therapies, usually in combination with chemotherapy, improved the cure rate in early-stage breast cancer and lengthened survival in the advanced setting (4).

A possible role of the immune system in fighting breast cancer has been supposed, however results have not been substantial. It has also become apparent that both innate and adaptive immunity display harmful features, promoting tumor progression as well as mediating tumor destruction (5). Thus, the inflammatory cell infiltration of tumors contributes either positively or negatively to tumor invasion, growth, metastasis and patient outcomes (6-8).

Recently, rising attention has been given to the degree of lymphocytic infiltrate by standard histology, in a large cohort of breast cancers, and the association between traditional pathologic factors, biomarkers expression and breast cancer molecular subtypes (9). Particularly, T cells present the most important immunological response into tumor growth in early stages of cancer. They become regulatory T cells (Tregs) after chronic stimulation, and interact with tumor cells, promoting rather than inhibiting cancer development and progression (10). In this context, this review proposed to discuss the prognostic value of T regulatory infiltration in microenvironment of HER2-enriched breast cancer.

HER2-enriched breast cancer

HER2 present different spellings, such as c-erb-2, cerbB-2, C-erbB-2, HER-2, HER-2/neu, ERBB2, erbB2, erbB-2, neu/c-erbB-2/oncogeneneu, neu protein, neu (11), although the common reference for this receptor is HER2. This proto-oncogene is located at 17q21 genetic locus, and encodes a 185 kDa transmembrane tyrosine kinase growth factor receptor (12).

The HER2 comes from a family of epidermal growth factor, the ErbB or epidermal growth factor (EGF) family of tyrosine kinase receptors, which includes four members: EGFR/ErbB1/HER1, ErbB2/Neu/HER2, ErbB3/HER3, and ErbB4/HER4. All ErbBs have a common extracellular ligand-binding domain, a single membrane-spanning region, and a cytoplasmic protein tyrosine kinase domain (13).

Ligand binding induces conformational changes and receptor homo/heterodimerization by the interaction of extracellular cysteine rich regions of ErbB receptors. This dimerization activates intracellular tyrosine kinase domain, promotes the auto phosphorylation of tyrosine residues of their cytoplasmic tails, and thus, triggers pathways that results in survival and cellular proliferation (14). However, it is known that HER2 is already in binding conformation even in the absence of any ligand. This fact could explain why no ligand for this receptor has been identified so far (15).

HER2 is found in normal breast epithelial cells, but is overexpressed in 20 to 30% of breast cancers, and since many years ago, is considered a marker of poor prognosis (16), since its overexpression is associated with an aggressive phenotype of tumor cells, resistance to anti-hormonal cytotoxic therapies and low overall survival. Furthermore, patients with HER2-overexpression and sentinel lymph node (SLN) commitment present higher involvement of non-sentinel lymph node metastases than other breast cancer subtypes (17).

Currently, numerous developments and refinements to the available technologies for HER2 testing have been achieved. However, the most widely used and the largest knowledge base techniques are the immunohistochemistry and FISH testing (18).

The management of aggressive and metastatic HER2-enriched breast cancer is dependent on the use of anti-HER2 therapies, such as trastuzumab (Herceptin®), a humanized monoclonal antibody, and lapatinib (Tykerb/Tyverb®), a tyrosine kinase inhibitor for HER1 and 2, which are associated with superior survival compared to patients treated with chemotherapy alone (19). However, a significant proportion of cases develop resistance to these therapies. Consequently, there is great urgency to detect these distinct entities and to develop therapies that will treat these tumors once they become resistant.

Moreover, new prognostic and predictive markers are essential to identify patients whose tumors are aggressive and at the highest risk for developing metastases, which might enable oncologists to assemble personalized treatment strategies to these patients.

Immune cells infiltration in tumor microenvironment

A complex stromal microenvironment nourishes tumor growth, involving fibroblasts, bone-marrow-derived cells, vascular and lymphatic vessels, cytokines and chemokines, and infiltrating immune cells, which support tumor progression (20).

During the past decade, new insights provided valuable knowledge related to the intrinsic mechanisms of dynamic interplay between immune cells and tumor progression. In this particular, the elicited immune response against a tumor may drive the outcome of an immune response (21). Furthermore, immune infiltrates are heterogeneous between tumor types, diverge substantially from patient to patient and can be located in different places of the tumor, such as the core (the center), the invasive margin or in the adjacent tertiary lymphoid structures (20).

All immune cell types may be found in a tumor, such as macrophages, dendritic cells, mast cells, natural killer (NK) cells, naïve and memory lymphocytes, B cells and effector T cells, including T helper 1 (T_H1) cell, T_H2, T_H17 and cytotoxic T cells (20). However, evidence suggest that, unlike cells found in lymphoid organs that productively respond to pathogens and non-self or altered molecules, immune cells in tumors are deregulated and functionally impaired. Thus, tumor masses can also contain regulatory T lymphocytes (Tregs), myeloid-derived suppressor cells (MDSC) and alternatively activated macrophages (22).

The most important immunological response in the early stages of cancer is mediated by T cells, especially the cytotoxic $CD8^+$ T lymphocytes (CTL), that might play a crucial role in the anti-tumor immunity, resulting in better clinical outcomes, such as better breast cancer patient survival (23, 24).

On the other hand, in the late stages of the cancer progression the T_H1 subset may switch to (Treg) phenotype, thus suppressing the tumor-associated antigen-specific immunity, promoting, rather than inhibiting, cancer development and progression, consequently conferring negative prognostic effects on breast cancer patient outcomes (10, 23, 25).

Regulatory T cells

Regulatory T cells were described in 1995 with reference to its involvement in immune response regulation and cellular activation (26). These cells can suppress effector T cell responses as well as the activity of other immune cells, such as mast cell, dendritic cells and B cells. For this reason, Tregs are key mediators of peripheral tolerance preventing undesirable immune responses. Many studies have provided strong evidence that Tregs may reside at diverse anatomical locations, and express different surface markers, among them: CD25 (IL-2 receptor α-chain or IL-2Rα), cytotoxic lymphocyte associated protein 4 (CTLA-4) and glucocorticoid-induced tumor necrosis factor (TNF) receptor (27, 28). Moreover, Tregs specifically express the forkhead box P3 (FOXP3), which encodes a transcription factor that plays a critical role in the differentiation, development, maintenance and function of this cell population (29-31).

Tregs may suppress immune cells by several mechanisms, such as (i) releasing granzymes and perforins, (ii) delivering a negative signal to T cells via up-regulation of intracellular cyclic AMP, which leads to inhibition of T cell proliferation and IL-2 formation, (iii) interacting with B7 expressed by responder T cells through the CTLA-4, and (iv) secreting suppressive cytokines, interleukin 10 (IL-10), interleukin 35 (IL-35) and transforming growth factor β (TGF-β) (32).

Although new subsets of these cells have been identified, two main populations of Treg cells have been broadly characterized: thymus-derived, or natural Tregs (nTregs) cells, and peripherally generated, or induced Tregs (iTregs). Both subsets have similar phenotypic characteristics and comparable suppressive function against T cell-mediated immune response and diseases. However, both FOXP3-positive Treg subsets exhibit some specific differences such as different mRNA transcripts and protein expression, epigenetic modification, and stability (33).

According to a review developed by Adeegbe and Nishikawa (34), the composition of Treg cells within tumor and/or circulation in human cancer patients can be nTreg, recruited to the tumor site and actively expanding, and/or a pool of iTreg derived from converted $CD4^+$ $CD25^-$ $FOXP3^-$ cells.

Studies have demonstrated that Treg recruitment into tumor occurs through a chemokine gradient, particularly of CCL22 and CCL1, through their respective receptors CCR4 and CCR8 (35, 36). Gobert and colleagues (37) observed that breast cancer-infiltrating Tregs express very low to undetectable CCR4 levels, and explained that this decreased CCR4

expression could result from internalization in vivo consecutive to an active recruitment through CCL22, highly produced by tumor cells.

Moreover, $CD4^+$ $CD25^-$ $FOXP3^-$ cells can be converted to iTreg directly or indirectly by TGF-β (38), which in tumor microenvironment is produced by several cell types, like cancer-associated fibroblast (CAFs) (39), tumor-associated macrophages, immature dendritic cells, MDSCs (40), tumor cells, and Treg themselves, generating sustained local immunosuppression.

Thus, as previously described, it has been shown that the immune system must participate in the control and elimination of tumor cells, and the presence of a lymphocytic infiltrate, particularly of Tregs, in cancer tissue is associated with clinical outcome (41-43). In this context, the efficient immune response against tumor is highly dependent, among several factors, on the lymphocyte subpopulation prevalent at the right place, and at the right time.

Treg infiltration in HER2-enriched breast tumor

As previously discussed, it is likely that Tregs may exert a prognostic impact in breast cancer, down regulating the immune response and inducing tolerance to the tumor, despite the presence of tumor antigen–specific T cells (44).

However, apparently the density of intratumoral and peritumoral Treg infiltration are independent prognostic factors. Demir et al. (45) established a predictive and prognostic effect of intratumoral Tregs in locally advanced breast cancer patients. These authors found that patients with high intratumoral tumor-infiltrating Tregs before chemotherapy had a significantly shorter overall survival than patients with low Treg infiltrates. In accordance with these authors, Liu and colleagues (46) also associated the intratumoral Treg infiltration with decreased overall survival, and furthermore with other unfavorable features, like decreased progression-free survival and high histological grade. Contrariwise, Gobert et al. (37) found that the presence of Tregs surrounding the tumor, but not within the tumor itself, is associated to a higher risk of relapse and death.

The Treg infiltration has also been studied in sentinel lymph nodes with and without metastatic breast carcinoma, since SLN are important sites of immunomodulation. Although the number of Tregs in SLN were not correlated with metastases, it was correlated with the size of primary breast invasive ductal carcinoma (47, 48).

Thus, the prognostic value of Treg infiltrate seems to depend significantly on their location, but possibly it is also dependent on molecular subtype, suggesting that the biologic properties of Treg are influenced by the tumor microenvironment in which they reside (49).

Although Bohling and Allison (25) found a tendency to association between Treg infiltrates with triple-negative breast cancer subtype (negative to hormone receptors and HER2-overexpression), some studies have demonstrated the opposite, i.e. association between tumor-infiltrating Treg with hormone receptors negativity and HER2- overexpression (45, 46, 50).

In tumors where an association between Treg infiltration and HER-overexpression was found, Tregs were mainly correlated with unfavorable prognostics, such as higher tumor grade, decreased overall survival and progression-free survival (46, 50). In contrast, Tsang et al. (9) found that lymphocytic infiltration was correlated with smaller tumor size, a good prognostic feature in HER2-enriched tumors. However, these authors considered both cytotoxic $CD8^+$ T lymphocytes and Tregs, and observed only a correlation between this breast cancer subtype and the CTL, an important constituent of anti-tumor immunity, which could explain why the lymphocytic infiltrate was associated with a better prognosis. The prognostic impact of intratumoral or peritumoral lymphocyte infiltration in HER2-enriched BC is presented in the Table 1.

Table 1. Prognostic impact of lymphocyte infiltration in HER2-enriched breast cancer microenvironment.

Lymphocyte	Location	Prognostic
Regulatory T cell	Intratumoral	Shorter overall survival (33,45,50)
		Decreased progression-free survival and high histological grade (51)
		No clinical associations (52-54)
	Peritumoral	Reduced disease-free interval and higher risk of relapse and death (37)
$CD8^+$ T cell	Intratumoral	Smaller tumor size (9)
		Low mortality and relapse (23)
		Reduced mortality hazard (55)
		Pathologic complete responses (44)
	Peritumoral	Reduced hazard of dying (55)

The higher numbers of regulatory T cells in the HER2-enriched breast tumors can be explained, at least partially, by the repertoire of chemokines and cytokines present in their microenvironment. As described above, Tregs may be recruited to the tumor

microenvironment through a gradient of chemokines, like the CCL22, which according Li and colleagues (51), is highly expressed in HER2-enriched breast tumors. Moreover this breast cancer subtype presents high plasmatic levels and tumor expression of TGF-β, which is produced by several cell types in tumor bed, including Tregs (51, 52).

In early stages of breast cancer, the TGF-β inhibits epithelial cell cycle progression and promotes apoptosis, showing tumor suppressive effects. However, in late stages, this cytokine is linked with increased tumor progression, higher cell motility, cancer invasiveness and metastasis (53).

A functional synergy between TGF-β and HER2 has been characterized in breast cancer models (in vitro and in vivo). TGF-β1 and TGF-β3 cooperate with HER2 in inducing cell motility and invasion, and evidences suggest that blockage of HER2:TGF-β crosstalk may significantly enhance the efficiency of conventional therapies in breast cancer patients with HER2-overexpression (54-56)

Furthermore, this cytokine is also involved in the differentiation of $CD4^+$ $CD25^-$ $FOXP3^-$ T cells to Treg in models of different types of cancer (38, 57, 58), contributing to immunosuppression of tumor microenvironment.

As discussed in the present review, it becomes clear the profound influence that immune cells infiltration, particularly of Tregs, can promote on breast cancer prognosis. Data collected from large cohorts of human cancer demonstrated that the immune-classification has a prognostic value that may be superior to the Tumor staging (AJCC/UICC-TNM classification). Thus, it is imperative to begin incorporating immune scoring as a prognostic factor and to introduce this parameter as a marker to classify cancer, as a part of the routine diagnostic and prognostic assessment of tumors (59). Particularly in the current era, when immunotherapies are progressively reaching the clinics and improving cancer treatment (60), characterization of immune profile predominating in cancer subtypes may provide therapeutic targets and predict those patients whose might be benefited by these several emerging treatments.

Ultimately, it is known that the amplification of HER2 oncogene is a relevant prognostic factor for breast cancer, besides many patients develop treatment resistance, demonstrating the need for new markers emergence, even for this molecular subtype that have a particular target therapy. Within this context, the importance of the immune system to tumor microenvironment is crucial, focusing on the subset of regulatory T cells, whose infiltration into tumor microenvironment appears to have important prognostic implications.

Conclusion

Although the focus of this review has been the prognostic value of regulatory T cell infiltration in HER2-enriched breast cancer, other immune cells must be considered, for example the cytotoxic $CD8^+$ T cells, which play a crucial role in anti-tumor immunity. Furthermore, no standard cutoff point for T lymphocytes infiltration was found in literature, what may have caused discrepant results, highlighting the importance of further studies to standardize cutoff scores. Finally, future prospects are needed to define the relevance of lymphocytes density in intratumoral and peritumoral locations, which appears to be independent prognostic factors.

Acknowledgments: We acknowledge the Conselho Nacional de Desenvolvimento Científico e Tecnológico - CNPq, Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, the Fundação Araucária and Coordenadoria de Pós-Graduação, State University of Londrina - PROPPG-UEL.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References

1. Banin Hirata BK, Oda JM, Losi Guembarovski R, Ariza CB, de Oliveira CE, Watanabe MA. Molecular markers for breast cancer: prediction on tumor behavior. Disease markers. 2014;2014:513158. Epub 2014/03/05.
2. Presson AP, Yoon NK, Bagryanova L, Mah V, Alavi M, Maresh EL, et al. Protein expression based multimarker analysis of breast cancer samples. BMC cancer. 2011;11:230. Epub 2011/06/10.
3. Weigelt B, Reis-Filho JS. Molecular profiling currently offers no more than tumour morphology and basic immunohistochemistry. Breast cancer research : BCR. 2010;12 Suppl 4:S5. Epub 2011/01/05.
4. Kim M, Agarwal S, Tripathy D. Updates on the treatment of human epidermal growth factor receptor type 2-positive breast cancer. Current opinion in obstetrics & gynecology. 2014;26(1):27-33. Epub 2013/12/18.

5. Kundu N, Ma X, Holt D, Goloubeva O, Ostrand-Rosenberg S, Fulton AM. Antagonism of the prostaglandin E receptor EP4 inhibits metastasis and enhances NK function. Breast cancer research and treatment. 2009;117(2):235-42. Epub 2008/09/17.

6. Ostrand-Rosenberg S. Cancer and complement. Nature biotechnology. 2008;26(12):1348-9. Epub 2008/12/09.

7. Pages F, Berger A, Camus M, Sanchez-Cabo F, Costes A, Molidor R, et al. Effector memory T cells, early metastasis, and survival in colorectal cancer. The New England journal of medicine. 2005;353(25):2654-66. Epub 2005/12/24.

8. Zhang L, Conejo-Garcia JR, Katsaros D, Gimotty PA, Massobrio M, Regnani G, et al. Intratumoral T cells, recurrence, and survival in epithelial ovarian cancer. The New England journal of medicine. 2003;348(3):203-13. Epub 2003/01/17.

9. Tsang JY, Hui SW, Ni YB, Chan SK, Yamaguchi R, Kwong A, et al. Lymphocytic infiltrate is associated with favorable biomarkers profile in HER2-overexpressing breast cancers and adverse biomarker profile in ER-positive breast cancers. Breast cancer research and treatment. 2014;143(1):1-9. Epub 2013/11/26.

10. Lee S, Cho EY, Park YH, Ahn JS, Im YH. Prognostic impact of FOXP3 expression in triple-negative breast cancer. Acta Oncol. 2013;52(1):73-81. Epub 2012/10/19.

11. Eisenberg ALA, Koifman S. Cancer de mama: Marcadores tumorais. Revista Brasileira de Cancerologia. 2001;47(4):11.

12. Ross JS, Fletcher JA. The HER-2/neu oncogene in breast cancer: prognostic factor, predictive factor, and target for therapy. Stem Cells. 1998;16(6):413-28. Epub 1998/12/01.

13. Holbro T, Civenni G, Hynes NE. The ErbB receptors and their role in cancer progression. Exp Cell Res. 2003;284(1):99-110.

14. Citri A, Skaria KB, Yarden Y. The deaf and the dumb: the biology of ErbB-2 and ErbB-3. Exp Cell Res. 2003;284(1):54-65.

15. Lonardo F, Di Marco E, King CR, Pierce JH, Segatto O, Aaronson SA, et al. The normal erB-2 product is an atypical receptor-like tyrosine kinase with constitutive activity in the absense of ligand. The New Biologist. 1990;2(11):11.

16. Rubin I, Yarden Y. The basic biology of HER2. Ann Oncol. 2001;12:3-8.

17. Gulben K, Berberoglu U, Aydogan O, Kinas V. Subtype is a predictive factor of nonsentinel lymph node involvement in sentinel node-positive breast cancer patients. Journal of breast cancer. 2014;17(4):370-5. Epub 2014/12/31.

18. Pathmanathan N, Bilous AM. HER2 testing in breast cancer: an overview of current techniques and recent developments. Pathology. 2012;44(7):587-95. Epub 2012/11/01.

19. Knutson KL, Perez EA, Ballman KV, Erskine CL, Fox N, McCarl C-A, et al., editors. Generation of adaptive HER2-specific immunity in HER2 breast cancer patients by addition of trastuzumab to chemotherapy in the adjuvant setting: NCCTG (Alliance) study N9831. J Clin Oncol (Meeting Abstracts); 2013.

20. Fridman WH, Pages F, Sautes-Fridman C, Galon J. The immune contexture in human tumours: impact on clinical outcome. Nat Rev Cancer. 2012;12(4):298-306. Epub 2012/03/16.

21. DeNardo DG, Coussens LM. Inflammation and breast cancer. Balancing immune response: crosstalk between adaptive and innate immune cells during breast cancer progression. Breast cancer research : BCR. 2007;9(4):212. Epub 2007/08/21.

22. Kerkar SP, Restifo NP. Cellular constituents of immune escape within the tumor microenvironment. Cancer research. 2012;72(13):3125-30. Epub 2012/06/23.

23. Feng Y, Huang R, He Y, Lu A, Fan Z, Fan T, et al. Efficacy of physical examination, ultrasound, and ultrasound combined with fine-needle aspiration for axilla staging of primary breast cancer. Breast cancer research and treatment. 2015;149(3):761-5. Epub 2015/02/11.

24. Mahmoud SM, Paish EC, Powe DG, Macmillan RD, Grainge MJ, Lee AH, et al. Tumor-infiltrating CD8+ lymphocytes predict clinical outcome in breast cancer. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2011;29(15):1949-55. Epub 2011/04/13.

25. Bohling SD, Allison KH. Immunosuppressive regulatory T cells are associated with aggressive breast cancer phenotypes: a potential therapeutic target. Modern pathology : an official journal of the United States and Canadian Academy of Pathology, Inc. 2008;21(12):1527-32. Epub 2008/09/30.

26. Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. J Immunol. 1995;155(3):1151-64. Epub 1995/08/01.

27. Sakaguchi S. Naturally arising Foxp3-expressing CD25+CD4+ regulatory T cells in immunological tolerance to self and non-self. Nature immunology. 2005;6(4):345-52. Epub 2005/03/24.

28. Schmetterer KG, Neunkirchner A, Pickl WF. Naturally occurring regulatory T cells: markers, mechanisms, and manipulation. Faseb J. 2012;26(6):2253-76. Epub 2012/03/01.

29. Fontenot JD, Gavin MA, Rudensky AY. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. Nature immunology. 2003;4(4):330-6. Epub 2003/03/04.

30. Gavin MA, Rasmussen JP, Fontenot JD, Vasta V, Manganiello VC, Beavo JA, et al. Foxp3-dependent programme of regulatory T-cell differentiation. Nature. 2007;445(7129):771-5. Epub 2007/01/16.

31. Zheng Y, Josefowicz SZ, Kas A, Chu TT, Gavin MA, Rudensky AY. Genome-wide analysis of Foxp3 target genes in developing and mature regulatory T cells. Nature. 2007;445(7130):936-40. Epub 2007/01/24.

32. Sakaguchi S, Wing K, Onishi Y, Prieto-Martin P, Yamaguchi T. Regulatory T cells: how do they suppress immune responses? International immunology. 2009;21(10):1105-11. Epub 2009/09/10.

33. Lin X, Chen M, Liu Y, Guo Z, He X, Brand D, et al. Advances in distinguishing natural from induced Foxp3(+) regulatory T cells. International journal of clinical and experimental pathology. 2013;6(2):116-23. Epub 2013/01/19.

34. Adeegbe DO, Nishikawa H. Natural and induced T regulatory cells in cancer. Frontiers in immunology. 2013;4:190. Epub 2013/07/23.

35. Fan X, Allison JP. Chemokines and recruitment of regulatory T cells to the tumor. The Journal of Immunoloy. 2009;182(40.42).

36. Curiel TJ, Coukos G, Zou LH, Alvarez X, Cheng P, Mottram P, et al. Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. Nat Med. 2004;10(9):942-9.

37. Gobert M, Treilleux I, Bendriss-Vermare N, Bachelot T, Goddard-Leon S, Arfi V, et al. Regulatory T Cells Recruited through CCL22/CCR4 Are Selectively Activated in Lymphoid Infiltrates Surrounding Primary Breast Tumors and Lead to an Adverse Clinical Outcome. Cancer Research. 2009;69(5):2000-9.

38. Liu VC, Wong LY, Jang T, Shah AH, Park I, Yang X, et al. Tumor evasion of the immune system by converting CD4+CD25- T cells into CD4+CD25+ T regulatory cells: role of tumor-derived TGF-beta. J Immunol. 2007;178(5):2883-92. Epub 2007/02/22.

39. Yu Y, Xiao CH, Tan LD, Wang QS, Li XQ, Feng YM. Cancer-associated fibroblasts induce epithelial-mesenchymal transition of breast cancer cells through paracrine TGF-beta signalling. British journal of cancer. 2014;110(3):724-32. Epub 2013/12/18.

40. Gabrilovich DI, Ostrand-Rosenberg S, Bronte V. Coordinated regulation of myeloid cells by tumours. Nature reviews Immunology. 2012;12(4):253-68. Epub 2012/03/23.

41. Bates GJ, Fox SB, Han C, Leek RD, Garcia JF, Harris AL, et al. Quantification of regulatory T cells enables the identification of high-risk breast cancer patients and those at

risk of late relapse. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2006;24(34):5373-80. Epub 2006/12/01.

42. Denkert C, Loibl S, Noske A, Roller M, Muller BM, Komor M, et al. Tumor-associated lymphocytes as an independent predictor of response to neoadjuvant chemotherapy in breast cancer. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2010;28(1):105-13. Epub 2009/11/18.

43. Schmidt M, Bohm D, von Torne C, Steiner E, Puhl A, Pilch H, et al. The humoral immune system has a key prognostic impact in node-negative breast cancer. Cancer research. 2008;68(13):5405-13. Epub 2008/07/03.

44. Ladoire S, Arnould L, Apetoh L, Coudert B, Martin F, Chauffert B, et al. Pathologic complete response to neoadjuvant chemotherapy of breast carcinoma is associated with the disappearance of tumor-infiltrating foxp3+ regulatory T cells. Clinical cancer research : an official journal of the American Association for Cancer Research. 2008;14(8):2413-20. Epub 2008/04/17.

45. Demir L, Yigit S, Ellidokuz H, Erten C, Somali I, Kucukzeybek Y, et al. Predictive and prognostic factors in locally advanced breast cancer: effect of intratumoral FOXP3+ Tregs. Clinical & experimental metastasis. 2013;30(8):1047-62. Epub 2013/07/10.

46. Liu F, Lang R, Zhao J, Zhang X, Pringle GA, Fan Y, et al. CD8(+) cytotoxic T cell and FOXP3(+) regulatory T cell infiltration in relation to breast cancer survival and molecular subtypes. Breast cancer research and treatment. 2011;130(2):645-55. Epub 2011/07/01.

47. Gupta R, Babb JS, Singh B, Chiriboga L, Liebes L, Adams S, et al. The numbers of FoxP3+ lymphocytes in sentinel lymph nodes of breast cancer patients correlate with primary tumor size but not nodal status. Cancer investigation. 2011;29(6):419-25. Epub 2011/06/09.

48. Gokmen-Polar Y, Thorat MA, Sojitra P, Saxena R, Badve S. FOXP3 expression and nodal metastasis of breast cancer. Cell Oncol (Dordr). 2013;36(5):405-9. Epub 2013/09/03.

49. deLeeuw RJ, Kost SE, Kakal JA, Nelson BH. The prognostic value of FoxP3+ tumor-infiltrating lymphocytes in cancer: a critical review of the literature. Clin Cancer Res. 2012;18(11):3022-9. Epub 2012/04/19.

50. Mahmoud SM, Paish EC, Powe DG, Macmillan RD, Lee AH, Ellis IO, et al. An evaluation of the clinical significance of FOXP3+ infiltrating cells in human breast cancer. Breast cancer research and treatment. 2011;127(1):99-108. Epub 2010/06/18.

51. Li YQ, Liu FF, Zhang XM, Guo XJ, Ren MJ, Fu L. Tumor secretion of CCL22 activates intratumoral Treg infiltration and is independent prognostic predictor of breast cancer. PloS one. 2013;8(10):e76379. Epub 2013/10/15.

52. Herrera AC, Panis C, Victorino VJ, Campos FC, Colado-Simao AN, Cecchini AL, et al. Molecular subtype is determinant on inflammatory status and immunological profile from invasive breast cancer patients. Cancer immunology, immunotherapy : CII. 2012;61(11):2193-201. Epub 2012/05/24.

53. Zarzynska JM. Two faces of TGF-beta1 in breast cancer. Mediators of inflammation. 2014;2014:141747. Epub 2014/06/04.

54. Wang SE. The functional crosstalk between HER2 tyrosine kinase and TGF-B signaling in breast cancer malignancy. Journal of Signal Transduction 2011;2011:8.

55. Seton-Rogers SE, Lu Y, Hines LM, Koundinya M, LaBaer J, Muthuswamy SK, et al. Cooperation of the ErbB2 receptor and transforming growth factor beta in induction of migration and invasion in mammary epithelial cells. P Natl Acad Sci USA. 2004;101(5):1257-62. Epub 2004/01/24.

56. Chow A, Arteaga CL, Wang SE. When tumor suppressor TGFbeta meets the HER2 (ERBB2) oncogene. Journal of mammary gland biology and neoplasia. 2011;16(2):81-8. Epub 2011/05/19.

57. Shen Y, Wei Y, Wang Z, Jing Y, He H, Yuan J, et al. TGF-beta regulates hepatocellular carcinoma progression by inducing Treg cell polarization. Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology. 2015;35(4):1623-32. Epub 2015/04/01.

58. Moo-Young TA, Larson JW, Belt BA, Tan MC, Hawkins WG, Eberlein TJ, et al. Tumor-derived TGF-beta mediates conversion of CD4+Foxp3+ regulatory T cells in a murine model of pancreas cancer. J Immunother. 2009;32(1):12-21. Epub 2009/03/25.

59. Galon J, Pages F, Marincola FM, Thurin M, Trinchieri G, Fox BA, et al. The immune score as a new possible approach for the classification of cancer. Journal of translational medicine. 2012;10:1. Epub 2012/01/05.

60. Bluestone JA, Tang Q. Immunotherapy: making the case for precision medicine. Science translational medicine. 2015;7(280):280ed3. Epub 2015/03/27.

ARTIGO 2

FOXP3 allelic variants and haplotypes are associated with susceptibility and prognosis in aggressive breast cancer subtypes

Abstract

The present study aimed to evaluate two *FOXP3* single nucleotide polymorphisms (SNP), g.10403A>G (rs2232365) and g.8048A>C (rs3761548), in 117 breast cancer (BC) samples, including, Luminal B HER2+ (LB), HER2-enriched (HER2+) and triple-negative (TN), and in 300 neoplasia-free controls. It was observed a significant association between AA genotype of g.10403A>G in relation to BC susceptibility (p=0.046). GG genotype of g.10403A>G polymorphism was correlated with higher proliferation index Ki-67 in HER2+ subtype (p=0.019; τ=-0.47) and higher staging in TN (p=0.032; τ=-0.23). It was also found correlation between AA genotype of g.8048A>C and higher Ki-67 (p=0.018; τ=0.47) and lower histological grade (p=0.026; τ=-0.39), in HER2+ subtype. Although no significant difference was observed in haplotype distribution between controls and BC patients, analysis for clinical outcome showed significant correlation of GA haplotype with lower histological grade (p=0.009; τ=-0.15) and higher Ki-67 (p=0.036; τ=0.43) in HER2+ subtype, and higher staging in TN (p=0.044; τ=0.29). On the other hand, the AC haplotype was correlated with lesser proliferation index Ki-67 (p=0.005; τ=-0.54) and TNM staging (p=0.027; τ=-0.29) in HER2+ and TN subtypes, respectively. Our results showed that *FOXP3* influence regarding clinical outcome depends greatly of BC subtypes and indicate this transcription factor as a promising susceptibility and prognosis marker in aggressive BC subtypes.

Keywords: FOXP3, genetic polymorphisms, haplotypes, breast cancer.

Introduction

The National Cancer Institute (INCA) estimated 57,960 new cases of breast cancer (BC) for 2016 and 2017 in Brazil. It is worth noting that, regardless of non-melanoma skin cancer, the mammary tumor is the most common among women in many regions for the country, accounting high morbidity and mortality (INCA 2016).

BC represents a complex and heterogeneous disease that comprises distinct pathologies, histological features, and clinical outcome. The status of estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor type 2 (HER2) and proliferation index Ki-67 has been used as predictive markers to identify a high-risk phenotype and for selection of most efficient therapies (Weigelt, Mackay et al. 2010; Presson, Yoon et al. 2011; Goldhirsch, Winer et al. 2013). These molecular markers also are, generally, used to classify the BC in subtypes, named Luminal A, Luminal B (LB), HER2-enriched (HER2+) and basal-like, also termed as triple-negative (TN) (TCGA 2012). Among these subtypes, the basal-like have the worst prognosis, while Luminal A have the best (Cho 2016). Within the tumors with HER2 overexpression, the Luminal B (hormonal receptors positive) were associated with better prognosis compared with the HER2-enriched subtype (Coates, Winer et al. 2015).

Forkhead box P3 (FOXP3) is an essential transcription factor to development and functions of Regulatory T cells (Tregs) (Fontenot, Gavin et al. 2003). Increased levels of $FOXP3^{+}$ Tregs in peripheral blood and tumor microenvironment have been reported in different types of cancer, including breast one (Bates, Fox et al. 2006). These cells play an important role in effector immune responses suppression, thus may contribute to tumorigenesis.

The accumulation of Tregs in local lymph nodes or in tumors is associated with a less favorable prognosis (Ladoire, Arnould et al. 2008; Leffers, Gooden et al. 2009). Although Tregs are the major cell type expressing FOXP3, it has been demonstrated that the tumor cell itself can express this protein, such as pancreatic cancer (Hinz, Pagerols-Raluy et al. 2007), melanoma (Ebert, Tan et al. 2008) and breast tumors (Ladoire, Arnould et al. 2011; Lopes, Guembarovski et al. 2014). Moreover FOXP3 expression in tumor cells could be an independent strong prognostic factor for distant metastasis in BC (Merlo, Casalini et al. 2009), in contrast with these data, this transcription factor was also demonstrated to be a tumor suppressor gene, acting as a transcriptional repressor of *SKP2* and *HER2*, two important BC oncogenes (Zuo, Liu et al. 2007; Zuo, Wang et al. 2007).

Regarding FOXP3 dual role in tumor microenvironment, investigation of polymorphisms and possible associations with cancer may shed light on the molecular pathogenesis of disease and opened new windows to susceptible individuals screening (Fazelzadeh Haghighi, Ali Ghayumi et al. 2015). Polymorphisms in the *FOXP3* gene may change it quantitatively or functionally, thereby contributing to an immune imbalance in cancer. To date, *FOXP3* allelic variants have been associated with a variety of immune-related diseases, such as allergic rhinitis (Fodor, Garaczi et al. 2011), idiopathic infertility and endometriosis-related infertility (Andre, Barbosa et al. 2011). Furthermore *FOXP3* polymorphisms have also been associated

with different types of cancer, such as Wilm's tumor (Ozawa, Ariza et al. 2016), hepatocellular (Chen, Zhang et al. 2013), colorectal (Chen, Yu et al. 2014) and non-small cell lung carcinoma (He, Bo et al. 2013). However, few studies, have investigated BC patients (Jahan, Ramachander et al. 2014; Jiang and Ruan 2014), especially in its molecular subtypes and in relation to its clinical outcomes. In this context, the present study aimed to investigate possible association between two *FOXP3* single nucleotide polymorphisms (SNPs), located in intronic region, with aggressive BC subtypes susceptibility and clinical outcome in a population from South region of Brazil.

Material and Methods

Ethics aspects and sample characterization

Human Ethics Committee of the State University of Londrina, Paraná, Brazil approved the study (CAAE-171231134000005231). Patients and controls were informed in detail regarding the research and the consent term was obtained. In the present study were included 107 peripheral blood samples (5 mL) collected with EDTA as anticoagulant and 10 paraffin-embedded tissues from patients attended in the Cancer Hospital of Londrina, Londrina, Paraná, Brazil (CHL). In total, were obtained 117 BC samples, of which 37 were diagnosed as Luminal B HER2+ (LB), 26 as HER2-enriched (HER2+) and 54 as triple-negative (TN) subtype.

For the control group, 300 blood samples were collected from women of same geographic region, without BC, proved by clinical and imaging examination, no self-declared BC family history or personal history of any malignant disease.

Clinicopathologic parameters data and immunohistochemical subgroups of BC were kindly provided by CHL. Prognostic parameters included: tumor size, lymph nodes commitment, Ki-67 proliferation index, histological grade and clinicopathological staging (Tumor/Node/Metastasis classification), which were determined according to the Union of International Control of Cancer classification criteria (Sobin, Gospodarowicz et al. 2009).

Genomic DNA extraction

Genomic DNA was obtained from peripheral blood cells using Biopur Mini Spin Plus Kit (Biometrix Diagnostica, Curitiba, Brazil), according to the manufacturer instructions. From the formalin fixed and paraffin-embedded samples, DNA was extracted using innuPREP DNA Mini (Analytik Jena, Jena, Germany), according to manufacturer's protocol. All

samples were quantified by NanoDrop 2000c®Spectrophotometer (Thermo Scientific, Wilmington, USA) at a wavelength of 260/280 nm and the final preparations were stored at -20ºC.

FOXP3 genotyping

Polymerase chain reaction (PCR) followed by enzymatic restriction (PCR-RLFP) was performed to genotype the SNPs rs2232365 and rs3761548, which also are termed g.10403A>G and g.8048A>C, respectively, according to GenBank Accession Number NG_007392.1.

For g.10403A>G and g.8048A>C genotyping were used specific primers according to Paradowska-Gorycka, Jurkowska et al. (2015) and He, Bo et al. (2013), respectively. The PCR was conducted using 1X of PCR Buffer (20mM of Tris-HCl ph 8.5; 50mM of KCl), 0.8 mM of $MgCl_2$, 0.1 mM of dNTP, 0.2μM of each primer, 1.25U of Taq DNA polymerase, 100 ng of genomic DNA and ultra-pure H_2O (Milli-Q) to complete a final volume of 25 μL. Negative controls were employed to make sure that no contaminants were introduced. The cycling protocol, used to both *FOXP3* polymorphisms, was a denaturation at 94ºC for 5 min, 35 cycles of 45 sec at 94ºC, 45 sec at 59ºC to g.10403A>G or 65ºC to g.8048A>C and 45 sec at 72ºC, and 10 min of final elongation at 72ºC. PCR products (5 μl) of g.10403A>G were digested overnight at 55ºC with 1 unit/reaction of *BsmBI* restriction endonuclease (New England Biolabs, Beverly, USA), and the PCR products (6 μl) of g.8048A>C were digested overnight at 37ºC with 2 units/reaction of *PstI* restriction endonuclease (New England Biolabs, Beverly, USA). All PCR and digested products were analyzed on polyacrylamide gel (10%), stained with silver nitrate. Primers sequences, restriction enzymes, and PCR and cleavage products are displayed in Table 1.

Table 1. Oligonucleotides sequences, fragment sizes and restriction products of g.10403A>G (rs2232365) and g.8048A>C (rs3761548) genetic polymorphisms.

	Oligonucleotide primers	PCR Products	Restriction enzyme	Restriction Products
g.10403A>G	F: 5' - AGGAGAAGGAGTGGGCATTT – 3' R: 5' - TGTGAGTGGAGGAGCTGAGG – 3'	249bp	BsmBI	Allele A: 249bp Alelle G: 132, 117bp
g.8048A>C	F: 5' - GGCAGAGTTGAAATCCAAGC – 3' R: 5' - CAACGTGTGAGAAGGCAGAA – 3'	155bp	PstI	Allele A: 155bp Allele C: 80, 75bp

F: forward; R: Reverse

Haplotype analysis

FOXP3 haplotypes were determined based on the genotypes of all study participants using PHASE software version 2.1.1 (Stephens, Smith et al. 2001; Stephens and Scheet 2005). Permutation test was also performed, using the same software, to check for haplotype distribution differences among controls and BC subgroups.

Statistical analysis

Binary logistic regression analyses were conducted to investigate associations between polymorphisms or haplotype structures and BC, controlled by age. Associations were tested considering genotypic models (heterozygotes or variant homozygotes versus wild homozygotes), dominant model (heterozygotes and variant homozygotes versus wild homozygotes) and recessive model (variant homozygotes versus wild homozygotes and heterozygotes). Correlation between polymorphisms or haplotypes structures and clinical parameters were assessed by Kendall-s tau-b rank correlation coefficient. All the statistical analyzes were performed in software SPSS 22.0 version (SPSS Inc., Chicago, USA), were two-tailed and with 5% significance level.

Results

In this study, the median age of BC patients was 51 (± 14) years and of control group was 55 (± 13) years (p=0.118). The prognostic parameters in general BC patients and in different subtypes are shown in Table 2, some characteristics were not available.

Table 2. Prognostic parameters in total BC and in aggressive subtypes.

Prognostic parameters		Total BC	LB	HER2+	TN
Tumor size	<1.5 cm	10 (8.9%)	3 (8.3%)	2 (8.7%)	5 (9.4%)
	1.5– 3.0cm	57 (50.9%)	23 (63.9%)	14 (60.9%)	20 (37.8%)
	>3.0 cm	45 (40.2%)	10 (27.8%)	7 (30.4%)	28 (52.8%)
TNM staging	I	19 (18.1%)	7 (20%)	5 (19.2%)	7 (15.9%)
	II	39 (37.2%)	14 (40%)	8 (30.8%)	17 (38.6%)
	III	37 (35.2%)	12 (34.3%)	9 (34.6%)	16 (36.4%)
	IV	10 (9.5%)	2 (5.7%)	4 (15.4%)	4 (9.1%)
Histological grade	II	30 (26.8%)	12 (33.3%)	8 (34.8%)	10 (18.9%)
	III	82 (73.2%)	24 (66.7%)	15 (65.2%)	43 (81.1%)
Ki-67	Low	7 (8.8%)	4 (19.0%)	0 (0.0%)	3 (7.3%)
	Moderate	25 (31.2%)	9 (42.9%)	7 (46.7%)	9 (23.6%)
	High	48 (60.0%)	8 (38.1%)	8 (53.3%)	32 (69.1%)
Lymph nodes commitment	No	54 (49.1%)	19 (52.8%)	12 (52.2%)	23 (45.1%)
	Yes	56 (50.9%)	17 (47.2%)	11 (47.8%)	28 (54.9%)

LB: Luminal B HER+; HER2+: HER2-enriched; TN: triple-negative.

Electrophoretic profiles of *FOXP3* polymorphisms are shown in Figure 1. Genotype distribution and allele frequencies for g.10403A>G and g.8048A>C polymorphisms are demonstrated in Table 3.

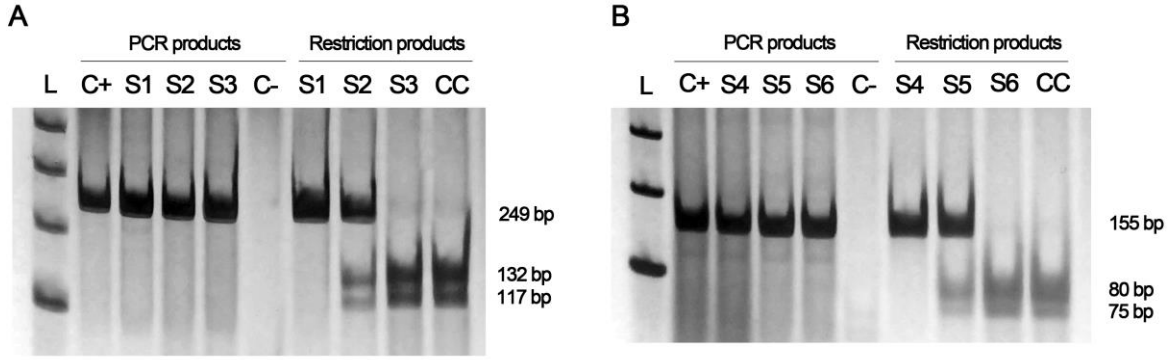

Figure 1. Eletrophoretic profiles of *FOXP3* polymorphisms. A. Electrophoretic profiles of g.10403A>G (rs2232365). B. Electrophoretic profiles of g.8048A>C (rs3761548). L: Ladder 100bp; C+: positive control; C-: negative control; CC: cleavage control; S1: homozygote genotype AA, S2: heterozygote genotype AG, S3: homozygote genotype GG; S4: homozygote genotype AA; S5: heterozygote genotype AC; S6: homozygote genotype CC.

Table 3. Allelic and genotypic frequencies of *FOXP3* polymorphisms g.10403A>G and g.8048A>C in total BC and aggressive subtypes

	Genotype	Controls (n=300)	Total BC (n=117)	LB (n=37)	HER2+ (n=26)	TN (n=54)
g.10403A>G (rs2232365)	AA	47 (15.7%)	26 (22.2%)	9 (24.3%)	5 (19.2%)	12 (22.2%)
	AG	147 (49.0%)	54 (46.2%)	15 (40.6%)	13 (50.0%)	26 (48.2%)
	GG	106 (35.3%)	37 (31.6%)	13 (35.1%)	8 (30.8%)	16 (29.6%)
	Allele A	40.2%	45.3%	44.6%	44.2%	46.3%
	Allele G	59.8%	54.7%	55.4%	55.8%	53.7%
g.8048A>C (rs3761548)	AA	41 (13.7%)	14 (12%)	7 (18.9%)	4 (15.4%)	3 (5.6%)
	AC	132 (44.0%)	48 (41%)	16 (43.3%)	10 (38.5%)	22 (40.7%)
	CC	127 (42.3%)	55 (47%)	14 (37.8%)	12 (46.1%)	29 (53.7%)
	Allele A	35.7%	32.5%	40.5%	34.6%	25.9%
	Allele C	64.3%	67.5%	59.5%	65.4%	74.1%

LB: Luminal B HER2+; HER2+: HER2-enriched; TN: triple negative.

In the present study, in total sample, AA genotype of g.10403A>G was associated with BC susceptibility (OR=1.93; CI 95% = 1.01 – 3.66; *p*=0.046) (Table 4).

Table 4. Case-control association study between *FOXP3* g.10403A>G and g.8048A>C genetic polymorphisms and total BC sample and aggressive subtypes.

	Model	Total BC [OR (CI)]	BC subtypes [OR(CI)]		
			LB (n=37)	HER2+ (n=26)	TN (n=54)
g.10403A>G	GG *vs* AG	1.22 (0.73 – 2.02) *p*=0.451	0.96 (0.43 - 2.16) *p*=0.92	1.32 (0.52 - 3.36) *p*=0.56	1.44 (0.71 - 2.91) *p*=0.31
	GG *vs* AA	1.93 (1.01 – 3.66) *p*=0.046*	1.92 (0.76 – 4.87) *p*=0.17	1.68 (0.51 - 5.65) *p*=0.39	2.15 (0.89 – 5.18) *p*=0.09
	[a]Dominant	1.38 (0.86 – 2.23) *p*=0.183	1.22 (0.58 - 2.57) *p*=0.61	1.35 (0.56 – 3.27) *p*=0.50	1.57 (0.81 - 3.05) *p*=0.18
	[b]Recessive	0.65 (0.35-1.05) *p*=0.07	0.52 (0.22-1.22) *p*= 0.13	0.74 (0.26-2.09) *p*=0.57	0.60 (0.29 - 1.25) *p*=0.17
g.8048A>C	CC *vs* AC	0.74 (0.46-1.19) *p*=0.22	0.85 (0.38 – 1.89) *p*=0.69	0.71 (0.29 - 1.77) *p*=0.46	0.69 (0.37 - 1.29) *p*=0.24
	CC *vs* AA	0.79 (0.39-1.60) *p*=0.52	1.52 (0.55 - 4.16) *p*=0.42	1.05 (0.31 - 3.30) *p*=1.05	0.29 (0.08-1.04) *p*=0.29
	[c]Dominant	0.77 (0.49-1.19) *p*=0.24	1.02 (0.49 - 2.11) *p*=0.97	0.81 (0.36 - 1.86) *p*=0.62	0.59 (0.33 - 1.09) *p*=0.09
	[d]Recessive	1.07 (0.55-2.08) *p*=0.84	0.60 (0.24 - 1.51) *p*=0.28	0.84 (0.27 - 2.60) *p*=0.76	2.67 (0.79 - 9.08) *p*=0.12

*Values of $p<0.05$ were considered statistically significant. OR: Odds ratio, CI; confidence intervals; BC: breast cancer; HER2+: HER2-enriched; TN: triple-negative

[a]Dominant = GG *vs* (AG+AA); [b]Recessive = (GG + AG) *vs* AA

[c]Dominant = CC *vs* (AC+AA); [d]Recessive = (CC + AC) *vs* AA

The predominant haplotype was the AC, in controls and in all BC subgroups, while the less common was the AA. No significant difference was found in haplotype distribution between controls and BC patients in the general sample (p=0.52). No association between the different haplotypes and BC susceptibility was found, in total sample neither in different subtypes. Although, was observed a strong tendency of association between AC haplotype and BC protection in total sample (p=0.053) and TN subtype (p=0.08), as well as between AA haplotype and BC protection in LB subtype (p=0.06) (Table 5).

Table 5. Case-control association study between *FOXP3* haplotypes and total BC sample and aggressive subtypes.

Model	Total BC OR (CI)	BC subtypes [OR (CI)]		
		LB	HER2+	TN
AC Dominant[a]	0.76 (0.47–1.21) p=0.25	1.03 (0.50 – 2.12) p=0.94	0.84 (0.36 – 1.99) p=0.69	0.55 (0.28 – 1.07) p=0.08
AC Recessive[b]	0.58 (0.33 – 1.01) p=0.053	0.50 (0.21 – 1.17) p=0.11	0.71 (0.25 – 2.01) p=0.52	0.57 (0.27 – 1.19) p=0.14
AA Dominant[c]	0.55 (0.13 – 2.30) p=0.42	0.19 (0.03 – 1.05) p=0.06	0.41 (0.05 – 3.74) p=0.43	NC
GC Dominant[d]	1.30 (0.83 – 2.05) p=0.26	1.74 (0.82 – 3.68) p=0.15	1.34 (0.58 – 3.09) p=0.49	1.01(0.55 – 1.86) p=0.97
GC Recessive[e]	1.10 (0.44 – 2.71) p=0.84	2.49 (0.32 – 19.7) p=0.39	0.81 (0.18 – 3.74) p=0.79	0.93 (0.30 – 2.89) p=0.90
GA Dominant[f]	1.26 (0.81 – 1.96) p=0.31	0.96 (0.46 – 1.99) p=0.90	1.19 (0.52 – 2.71) p=0.69	1.62 (0.89 – 2.95) p=0.12
GA Recessive[g]	1.35 (0.66 – 2.78) p=0.41	0.89 (0.32 – 2.50) p=0.82	1.09 (0.31 – 3.83) p=0.89	2.53 (0.74 – 8.62) p=0.14

OR: Odds ratio, CI; confidence intervals; BC: breast cancer; LB: Luminal B HER2+; HER2+: HER2-enriched; TN: triple-negative; NC: No calculated because the group did not present the haplotype.
[a]AC Dominant = AA, GC and GA carriers *vs* AC carriers; [b]AC Recessive = AA, GC and GA carriers *vs* ACAC
[c]AA Dominant = AC, GC and GA carriers *vs* AA carriers; [d]GC Dominant = AC, AA and GA carriers *vs* GC carriers; [e]GC Recessive = AC, AA, and GA carriers *vs* GCGC; [f]GA Dominant = AC, AA, and GC carriers *vs* GA carriers; [g]GA Recessive = AC, AA and GC carriers *vs* GAGA.

The analysis considering clinical parameters showed a significant correlation between GG genotype of g.10403A>G polymorphism and higher proliferation index Ki-67 in HER2+ subtype (p=0.019; τ=-0.47) and higher staging in TN subtype (p=0.032; τ=-0.23). It was also found a significant correlation between AA genotype of g.8048A>C and higher Ki-67 (p=0.018; τ=0.47) and lower histological grade, in HER2+ subtype (p=0.026; τ=-0.39) (Table 6).

Table 6. Correlation analysis of *FOXP3* g.10403A>G and g.8048A>C polymorphisms with prognostic parameters in different total BC sample and aggressive subtypes.

	Clinical outcomes	**Total BC *p* (tau)**	**Breast cancer subtypes [*p* (tau)]**		
			LB	**HER2+**	**TN**
g.10403A>G	TNM Staging	0.173 (τ=-0.11)	0.608 (τ=-0.08)	0,835 (τ=0.04)	0.032 (τ=-0.23)*
	Tumor size	0.546 (τ=0.05)	0.885 (τ=-0.02)	0.778 (τ=-0.05)	0.328 (τ=0.12)
	Ki-67	0.270 (τ=-0,11)	0.837 (τ=-0.04)	0.019 (τ=-0.47)*	0.536 (τ=-0.08)
	Histological grade	0.268 (τ=0.10)	0.846 (τ=0.03)	0.061 (τ=0.36)	0.909 (τ=0.02)
	LP commitment	0.412 (τ=-0.74)	0.337 (τ=-0.15)	0.366 (τ=-0.172)	0.869 (τ=-0.22)
g.8048A>C	TNM Staging	0.966 (τ=0.003)	0.894 (τ=-0.02)	0.167 (τ=-0.23)	0.084 (τ=0.23)
	Tumor size	0.210 (τ=-0.10)	0.403 (τ=-0.11)	0.912 (τ=0.02)	0.489 (τ=-0.09)
	Ki-67	0.557 (τ=-0.06)	0.28 (τ=0.21)	0.018 (τ=0.47)*	0.708 (τ=-0.05)
	Histological grade	0.135 (τ=-0.15)	0.754 (τ=-0.06)	0.026 (τ=-0.39)*	0.927 (τ=-0.01)
	LP commitment	0.773 (τ=-0.26)	0.662 (τ=0.07)	1.0 (τ=-0.0)	0.636 (τ=-0.06)

Kendall's Tau test; *Value of p<0.05 was considered statistically significant. BC: breast cancer; LB: Luminal B HER2+; HER2+: HER2-enriched; TN: triple-negative; LP: lymph node.

The analysis of correlation between *FOXP3* haplotypes and clinical outcomes showed significant correlations of GA haplotype with lower histological grade (p=0.009; τ =-0.15) and higher Ki-67 (p=0.036; τ =0.43) in HER2+ subtype and higher staging in TN (p=0.044; τ=0.29). The AC haplotype was correlated with lower Ki-67 (p=0.005; τ =-0.54) and lower TNM staging (p=0.027; τ =-0.29) in HER2+ and TN subtypes, respectively (Table 7).

Table 7. *FOXP3* haplotypes correlation analysis in relation to prognostic parameters in total BC sample and aggressive subtypes.

	Clinical outcomes	Haplotypes		
		AC	GA	GC
Total BC	TNM Staging	0.07 (τ =-0.16)	0.885 (τ =0.01)	0.06 (τ =0.18)
	Histological grade	0.415 (τ =0.07)	0.07 (τ =-0.17)	0.377 (τ =0.08)
	Tumor size	0.572 (τ =0.05)	0.157 (τ =-0.12)	0.853 (τ =0.01)
	Ki-67 index	0.374 (τ =-0.09)	0.517 (τ =0.06)	0.809 (τ =0.03)
	LP commitment	0.309 (τ =-0.09)	0.825 (τ =0.02)	0.443 (τ =0.07)
LB	TNM Staging	0.256 (τ =-0.18)	0.656 (τ =-0.07)	0.330 (τ =0.17)
	Histological grade	0.771 (τ =-0.05)	0.597 (τ =-0.09)	0.392 (τ =0.13)
	Tumor size	0.836 (τ =-0.03)	0.491 (τ =-0.10)	0.257 (τ =0.17)
	Ki-67 index	0.970 (τ =-0.01)	0.118 (τ =0.25)	0.177 (τ =-0.26)
	LP commitment	0.135 (τ =-0.23)	0.972 (τ =0.01)	0.295 (τ =0.17)
HER2+	TNM Staging	0.875 (τ =0.03)	0.186 (τ =-0.24)	0.06 (τ =0.34)
	Histological grade	0.104 (τ =0.31)	0.009 (τ =-0.15)*	0.968 (τ =0.01)
	Tumor size	0.491 (τ =-13)	0.811 (τ =-0.04)	0.976 (τ =0.01)
	Ki-67 index	0.005 (τ =-0.54)*	0.036 (τ =0.43)*	0.876 (τ =-0.04)
	LP commitment	0.756 (τ =-0.06)	0.373 (τ =0.17)	0.955 (τ =-0.01)
TN	TNM Staging	0.027 (τ =-0.29)*	0.044 (τ =0.29)*	0.591 (τ =0.08)
	Histological grade	0.861 (τ =0.02)	0.705 (τ =-0.06)	0.750 (τ =0.04)
	Tumor size	0.315 (τ =0.13)	0.419 (τ =-0.10)	0.502 (τ =-0.09)
	Ki-67 index	0.632 (τ =-0.06)	0.708 (τ =-0.05)	0.533 (τ =0.09)
	LP commitment	0.883 (τ =-0.02)	0.913 (τ =-0.02)	0.833 (τ =0.03)

Kendall's Tau test; *Value of $p<0.05$ was considered statistically significant. LP: lymph node

Discussion

In the present study, we analyzed g.10403A>G and g.8048A>C polymorphisms, in 117 BC patients and in 300 neoplasia-free controls. The results of the present study indicated an association of AA homozygous genotype (g.10403A>G) with aggressive BC susceptibility (OR = 1.93, 95% CI = 1.01 to 3.66), suggesting that individuals who had inherited both copies of the variant allele are more susceptible for BC than individuals with other genotypes.

As far as we researched, there are no articles relating a significant association for g.10403A>G to BC susceptibility, but significant associations have been proposed with other diseases, such as autoimmune diseases (Song, Shen et al. 2012; Song, Wang et al. 2013), unexplained recurrent spontaneous abortion (Wu, You et al. 2011; Saxena, Misra et al. 2015) and autism spectrum disorders (Safari, Ghafouri-Fard et al. 2017).

Wu, You et al. (2012) performed an extensive search for transcriptional factor-binding sites and found that g.10403A>G SNP is located in a putative binding site for the transcription factor GATA-3. More importantly, only when the A allele exists, this transcription factor can bind the promoter region of *FOXP3*. According Wang, Su et al. (2011) defective function of both GATA-3 and FOXP3 itself led to ablation of Treg cells, suggesting that the combined function of GATA-3 and FOXP3 is essential for FOXP3 expression, highlighting the indispensable role of GATA-3 in regulating Treg cell function. In this context, the A allele of g.10403A>G may be associated with increased FOXP3 expression and consequently in the maintenance of Treg function, contributing to suppression of effector antitumor immune response, and possibly explaining the association between this variant polymorphism with increased breast cancer susceptibility.

In the present study, no association was found between g.8048A>C in relation to BC susceptibility, nor in general sample neither in different subtypes. Similar observations were made by Raskin, Rennert et al. (2009) in Israeli population, Zheng, Deng et al. (2013) in Han Chinese population and Jahan, Ramachander et al. (2014) in Indian population. Additionally, a meta-analysis performed by Jiang and Ruan (2014) indicated that g.8048A>C is not associated with BC, but with susceptibility to hepatocellular carcinoma and non-small cell lung cancer.

No significant association between different *FOXP3* haplotypes and BC susceptibility was observed, or in the general sample and neither in different subtypes. To date, there are no articles relating the g.10403A>G and g.8048A>C haplotypes in relation to BC susceptibility or clinical outcomes of this disease.

In addition to BC assessment, our results showed a significant correlation between g.10403A>G GG genotype and higher proliferation index Ki-67 in HER2+ subtype ($p=0.019$) and higher staging in TN subtype ($p=0.032$). To date, this is the first study that observed a correlation between g.10403A>G polymorphism and BC prognostic parameters.

As previously discussed the G allele may be related to lower expression of FOXP3 due the lost binding site to GATA-3. Many studies have shown that, in BC, *FOXP3* could be

considered a tumor suppressor gene, conferring a better prognosis (Douglass, Meeson et al. 2014; Zhang, Xu et al. 2015).

Despite *FOXP3* g.8048A>C may not be playing a role in predisposing the Brazilian women to BC, we report a significant correlation of AA genotype with higher Ki-67 and lower histological grade in HER2+ subtype. None correlation of this polymorphism with prognostic parameters was found in TN subtype, which is in accordance with a previous study developed by our research group (Lopes, Guembarovski et al. 2014).

Like the c g.10403A>G GG genotype, the c g.8048A>C AA also appears to be related to *FOXP3* lower expression. Shen, Chen et al. (2010) observed that psoriatic patients with g.8048A>C AA genotype have reduced *FOXP3* gene expression. These authors demonstrated that the C to A change causes binding loss to E47 and c-Myb transcription factors, leading to a defective transcription of *FOXP3* gene.

Furthermore, Jahan, Cheruvu et al. (2013) observed a highly significant association of g.8048A>C AA genotype with BC advanced stages (III and IV). In the present study no correlation with tumor stage was found, perhaps this discrepant result is due to the BC subtypes studied. These authors did not stratify the BC sample and, probably, included subtypes of better prognosis, such as Luminal A and Luminal B HER2-, unlike the present study that comprised only aggressive breast cancer subtypes.

In contrast, we also found correlation of g.8048A>C AA genotype with better prognosis like lower histological grade. Ohara, Yamaguchi et al. (2009), analyzed *FOXP3* expression in breast tumor by qRT-PCR, and observed a significant correlation with higher histological grade. These authors attributed the correlation with worse prognosis to Treg infiltration. Other study using immunohistochemistry technique also showed association of FOXP3 expression by tumor cells with higher histological grade (Kim, Koo et al. 2013). However, in this study all tumor samples showed cytoplasmic or both cytoplasmic and nuclear FOXP3 expression, suggesting frequent deregulation of FOXP3 localization and failure to translocate to the nucleus in breast cancer cells, and explaining the correlation with worse prognosis. In this context the correlation of g.8048A>C AA genotype with better prognosis may reflect the lower functional Treg infiltration in tumor bed.

Furthermore, significant correlations between *FOXP3* haplotypes and prognostic parameters were found. This study showed a correlation of AC with better prognosis, such as lower proliferation index and staging, in HER2+ and TN, respectively. As discussed above, the polymorphisms may affect the expression of gene, in this way AC haplotype may be related

with higher FOXP3 expression, possibly explaining the correlation with better prognosis, since this transcription factor is considered a tumor suppressor gene in BC.

In conclusion, the present study showed, for the first time, a significant association of *FOXP3* g.10403A>G with susceptibility and prognosis of aggressive BC. Although the g.8048A>C may not be associated with BC susceptibility, significant correlations with clinical outcomes were found. Furthermore, the present study showed different correlations with prognostic parameters in LB, HER2+ and TN, highlighting that the impact of allelic variants may depend on tumor subtype. Moreover, the dual role of *FOXP3,* participating in Treg cells development and function from one side and acting as a tumor modulator gene from other side should not be ignored.

Conflict of Interests

The authors declare that there are no conflicts of interest.

Acknowledgments

The authors would like to acknowledge the volunteers who made this study possible. This work was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Londrina State University Coordination for Post-Graduation (PROPPG-UEL). The authors wish to express their gratitude to Fundação Araucária do Paraná and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

References

Andre, G. M., C. P. Barbosa, et al. (2011). "Analysis of FOXP3 polymorphisms in infertile women with and without endometriosis." Fertil Steril **95**(7): 2223-2227.

Bates, G. J., S. B. Fox, et al. (2006). "Quantification of regulatory T cells enables the identification of high-risk breast cancer patients and those at risk of late relapse." J Clin Oncol **24**(34): 5373-5380.

Chen, L., Q. Yu, et al. (2014). "Association of FoxP3 rs3761548 polymorphism with susceptibility to colorectal cancer in the Chinese population." Med Oncol **31**(12): 374.

Chen, Y., H. Zhang, et al. (2013). "FOXP3 gene polymorphism is associated with hepatitis B-related hepatocellular carcinoma in China." J Exp Clin Cancer Res **32**: 39.

Cho, N. (2016). "Molecular subtypes and imaging phenotypes of breast cancer." Ultrasonography.

Coates, A. S., E. P. Winer, et al. (2015). "Tailoring therapies--improving the management of early breast cancer: St Gallen International Expert Consensus on the Primary Therapy of Early Breast Cancer 2015." Ann Oncol **26**(8): 1533-1546.

Douglass, S., A. P. Meeson, et al. (2014). "Breast cancer metastasis: demonstration that FOXP3 regulates CXCR4 expression and the response to CXCL12." J Pathol **234**(1): 74-85.

Ebert, L. M., B. S. Tan, et al. (2008). "The regulatory T cell-associated transcription factor FoxP3 is expressed by tumor cells." Cancer Res **68**(8): 3001-3009.

Fazelzadeh Haghighi, M., M. Ali Ghayumi, et al. (2015). "Investigation of FOXP3 genetic variations at positions -2383 C/T and IVS9+459 T/C in southern Iranian patients with lung carcinoma." Iran J Basic Med Sci **18**(5): 465-471.

Fodor, E., E. Garaczi, et al. (2011). "The rs3761548 polymorphism of FOXP3 is a protective genetic factor against allergic rhinitis in the Hungarian female population." Hum Immunol **72**(10): 926-929.

Fontenot, J. D., M. A. Gavin, et al. (2003). "Foxp3 programs the development and function of CD4+CD25+ regulatory T cells." Nat Immunol **4**(4): 330-336.

Goldhirsch, A., E. P. Winer, et al. (2013). "Personalizing the treatment of women with early breast cancer: highlights of the St Gallen International Expert Consensus on the Primary Therapy of Early Breast Cancer 2013." Ann Oncol **24**(9): 2206-2223.

He, Y. Q., Q. Bo, et al. (2013). "FoxP3 genetic variants and risk of non-small cell lung cancer in the Chinese Han population." Gene **531**(2): 422-425.

Hinz, S., L. Pagerols-Raluy, et al. (2007). "Foxp3 expression in pancreatic carcinoma cells as a novel mechanism of immune evasion in cancer." Cancer Res **67**(17): 8344-8350.

INCA (2016). Estimate 2016: Cancer Incidence in Brazil. Rio de Janeiro**:** 51.

Jahan, P., R. Cheruvu, et al. (2013). "Association of FOXP3 (rs3761548) promoter polymorphism with nondermatomal vitiligo: A study from India." J Am Acad Dermatol **69**(2): 262-266.

Jahan, P., V. R. Ramachander, et al. (2014). "Foxp3 promoter polymorphism (rs3761548) in breast cancer progression: a study from India." Tumour Biol **35**(4): 3785-3791.

Jiang, L. L. and L. W. Ruan (2014). "Association between FOXP3 promoter polymorphisms and cancer risk: A meta-analysis." Oncol Lett **8**(6): 2795-2799.

Kim, M. H., J. S. Koo, et al. (2013). "FOXP3 expression is related to high Ki-67 index and poor prognosis in lymph node-positive breast cancer patients." Oncology **85**(2): 128-136.

Ladoire, S., L. Arnould, et al. (2008). "Pathologic complete response to neoadjuvant chemotherapy of breast carcinoma is associated with the disappearance of tumor-infiltrating foxp3+ regulatory T cells." Clin Cancer Res **14**(8): 2413-2420.

Ladoire, S., L. Arnould, et al. (2011). "Presence of Foxp3 expression in tumor cells predicts better survival in HER2-overexpressing breast cancer patients treated with neoadjuvant chemotherapy." Breast Cancer Res Treat **125**(1): 65-72.

Leffers, N., M. J. Gooden, et al. (2009). "Prognostic significance of tumor-infiltrating T-lymphocytes in primary and metastatic lesions of advanced stage ovarian cancer." Cancer Immunol Immunother **58**(3): 449-459.

Lopes, L. F., R. L. Guembarovski, et al. (2014). "FOXP3 transcription factor: a candidate marker for susceptibility and prognosis in triple negative breast cancer." Biomed Res Int **2014**: 341654.

Lopes, L. F., R. L. Guembarovski, et al. (2014). "FOXP3 transcription factor: a candidate marker for susceptibility and prognosis in triple negative breast cancer." Biomed Research International **2014**: 1-7.

Merlo, A., P. Casalini, et al. (2009). "FOXP3 expression and overall survival in breast cancer." J Clin Oncol **27**(11): 1746-1752.

Ohara, M., Y. Yamaguchi, et al. (2009). "Possible involvement of regulatory T cells in tumor onset and progression in primary breast cancer." Cancer Immunol Immunother **58**(3): 441-447.

Ozawa, P. M., C. B. Ariza, et al. (2016). "Wilms' tumor susceptibility: possible involvement of FOXP3 and CXCL12 genes." Mol Cell Pediatr **3**(1): 36.

Paradowska-Gorycka, A., M. Jurkowska, et al. (2015). "Genetic polymorphisms of Foxp3 in patients with rheumatoid arthritis." J Rheumatol **42**(2): 170-180.

Presson, A. P., N. K. Yoon, et al. (2011). "Protein expression based multimarker analysis of breast cancer samples." BMC Cancer **11**: 230.

Raskin, L., G. Rennert, et al. (2009). "FOXP3 germline polymorphisms are not associated with risk of breast cancer." Cancer Genet Cytogenet **190**(1): 40-42.

Safari, M. R., S. Ghafouri-Fard, et al. (2017). "FOXP3 gene variations and susceptibility to autism: A case-control study." Gene **596**: 119-122.

Saxena, D., M. K. Misra, et al. (2015). "The transcription factor Forkhead Box P3 gene variants affect idiopathic recurrent pregnancy loss." Placenta **36**(2): 226-231.

Shen, Z., L. Chen, et al. (2010). "Intron-1 rs3761548 is related to the defective transcription of Foxp3 in psoriasis through abrogating E47/c-Myb binding." J Cell Mol Med **14**(1-2): 226-241.

Sobin, L. H., M. K. Gospodarowicz, et al. (2009). TNM classification of malignant tumours.

Song, P., X. W. Wang, et al. (2013). "Association between FOXP3 polymorphisms and vitiligo in a Han Chinese population." Br J Dermatol **169**(3): 571-578.

Song, Q. H., Z. Shen, et al. (2012). "An association study of single nucleotide polymorphisms of the FOXP3 intron-1 and the risk of Psoriasis vulgaris." Indian J Biochem Biophys **49**(1): 25-35.

Stephens, M. and P. Scheet (2005). "Accounting for decay of linkage disequilibrium in haplotype inference and missing-data imputation." Am J Hum Genet **76**(3): 449-462.

Stephens, M., N. J. Smith, et al. (2001). "A new statistical method for haplotype reconstruction from population data." Am J Hum Genet **68**(4): 978-989.

TCGA (2012). "Comprehensive molecular portraits of human breast tumours." Nature **490**(7418): 61-70.

Wang, Y., M. A. Su, et al. (2011). "An essential role of the transcription factor GATA-3 for the function of regulatory T cells." Immunity **35**(3): 337-348.

Weigelt, B., A. Mackay, et al. (2010). "Breast cancer molecular profiling with single sample predictors: a retrospective analysis." Lancet Oncol **11**(4): 339-349.

Wu, Z., Z. You, et al. (2012). "Association between functional polymorphisms of Foxp3 gene and the occurrence of unexplained recurrent spontaneous abortion in a Chinese Han population." Clin Dev Immunol **2012**: 896458.

Wu, Z. G., Z. S. You, et al. (2011). "[Study on association of functional polymorphisms in Foxp3 gene with the susceptibility to unexplained recurrent spontaneous abortion]." Zhonghua Fu Chan Ke Za Zhi **46**(10): 763-768.

Zhang, C., Y. Xu, et al. (2015). "FOXP3 suppresses breast cancer metastasis through downregulation of CD44." Int J Cancer **137**(6): 1279-1290.

Zheng, J., J. Deng, et al. (2013). "Heterozygous genetic variations of FOXP3 in Xp11.23 elevate breast cancer risk in Chinese population via skewed X-chromosome inactivation." Hum Mutat **34**(4): 619-628.

Zuo, T., R. Liu, et al. (2007). "FOXP3 is a novel transcriptional repressor for the breast cancer oncogene SKP2." J Clin Invest **117**(12): 3765-3773.

Zuo, T., L. Wang, et al. (2007). "FOXP3 is an X-linked breast cancer suppressor gene and an important repressor of the HER-2/ErbB2 oncogene." Cell **129**(7): 1275-1286.

ARTIGO 3

Genetic polymorphisms and expression of *FOXP3* mRNA in aggressive breast cancer subtypes

Abstract

FOXP3 genetic polymorphisms, as well as their expression level, have been associated with cancer development and prognosis of breast cancer (BC). The present study aimed to investigate the influence of two *FOXP3* polymorphisms, g.10403A>G (rs2232365) and g.8048A>C (rs3761548) on its mRNA expression in aggressive BC subtypes, including Luminal B HER2-positive (LB), HER2-enriched (HER2+) and triple-negative (TN), as well as to correlate with prognostic parameters. *FOXP3* genotyping was performed in 61 BC patients, by polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) and mRNA expression quantification by quantitative real time PCR (qRT-PCR). It was observed that LB presented 2.6 fold lesser *FOXP3* mRNA expression when compared to normal mammary gland, while HER2+ and TN presented a slightly higher expression (1.8 and 1.5 folds, respectively). BC patients with AC haplotype in homozygous presented 3.2 folds higher *FOXP3* mRNA expression in relation to normal mammary gland, showing a significant correlation (p=0.039; rho=0.27). The FOXP3 mRNA expression was not correlated with prognostic parameters, in general BC patients, or in different subtypes. Although no difference was found for genotypes, our results demonstrated that *FOXP3* AC haplotype may be involved in the gene transcription regulation.

Keywords: FOXP3, genetic polymorphism, haplotype, genic expression, breast cancer.

Introduction

Breast cancer (BC) is the second most common cancer in the world and, by far, the most frequent cancer among women. It is a heterogeneous and phenotypically complex disease, with several biological subtypes that present distinct behaviors and responses to therapy. Generally, the breast cancer is classified in four intrinsic subtypes, named Luminal A (LA), Luminal B (LB), HER2-enriched (HER2+) and basal-like, the last one also frequently termed as triple-negative (TN) (TCGA 2012).

There is compelling evidence that tumors can elicit an immune response in its own microenvironment, which generally is considered ineffective to eliminate cancer cells. However, in the last years, assumption has emerged demonstrating the importance of immune cells infiltration, such as lymphocytes and tumor-associated macrophages, in the clinical evolution of many cancers (de la Cruz-Merino, Barco-Sanchez et al. 2013). Regarding the significance of different T cell phenotypes, it is likely that their importance and predictive capacity varies depending on BC subtype and tumor microenvironment (Miyan, Schmidt-Mende et al. 2016). Tumor infiltrating lymphocytes are often found in TN or HER2+ cancers (Coates, Winer et al. 2015).

Increased levels of regulatory T lymphocytes (Tregs) in tumor microenvironment have been reported in different types of cancer, including breast (Bates, Fox et al. 2006). These cells play an important role in suppression of effector immune responses, thus may contribute to tumorigenesis. The development and functions of these cells depends of an essential transcription factor, termed forkhead box p3 (FOXP3) (Fontenot, Gavin et al. 2003; Hori, Nomura et al. 2003).

Initially, it was postulated that FOXP3 expression was thought to be restricted to hematopoietic tissues. However, although data are scarce, its expression in other tissues has also been observed, including human tumor cells (Karanikas, Speletas et al. 2008).

Recent data suggest that FOXP3 expression in tumor cells could be an independent strong prognostic factor for distant metastasis in BC (Merlo, Casalini et al. 2009), but in contrast with these data, it was recently demonstrated to be a tumor suppressor gene, acting as a transcriptional repressor of *SKP2* and *HER2*, two important breast cancer oncogenes (Zuo, Liu et al. 2007; Zuo, Wang et al. 2007).

Genetic polymorphisms in *FOXP3* gene may change it quantitatively or functionally, thereby contributing to an immune imbalance in cancer. In this context, the present study aimed to investigate the influence of two intronic *FOXP3* polymorphisms, g.10403A>G (rs2232365) and g.8048A>C (rs3761548) on its mRNA expression in breast cancer samples, focusing the aggressive subtypes, LB, HER2+ and TN, as well as to analyze the correlation of *FOXP3* mRNA expression with BC prognostic parameters.

Materials and Methods

Ethics aspects and sample characterization

Human Ethics Committee of the Londrina State University, Paraná, Brazil approved the study (CAAE-171231134000005231). Patients were informed in detail regarding the research and the consent term was obtained. The samples used in this study were obtained from patients attended in the Cancer Hospital of Londrina, Londrina, Paraná, Brazil (CHL) and included: peripheral blood (5mL), collected with EDTA as anticoagulant, to analyze genetic polymorphisms, and fresh tumor tissue, to analyze mRNA expression. In total, were obtained 61 breast cancer samples, of which 19 were diagnosed as LB, 15 as HER2+ and 27 as TN subtype.

Data relating to prognostic parameters and immunohistochemical subgroups of BC were kindly provided by CHL. The parameters included: tumor size, lymph nodes involvement, Ki-67 proliferation index, histological grade, hormonal receptors, HER2 overexpression and TNM staging (Tumor/Node/Metastasis classification), which were determined according to the Union of International Control of Cancer classification criteria (Sobin, Gospodarowicz et al. 2009).

Genomic DNA extraction

Genomic DNA was obtained from peripheral blood cells using Biopur Mini Spin Plus Kit (Biometrix Diagnostica, Curitiba, Parana, Brazil), according to the manufacturer instructions. The DNA was ressuspended in 50 μL of elution buffer. All samples were quantified by NanoDrop 2000c®Spectrophotometer (Thermo Scientific, Wilmington, Delaware, USA) at a wavelength of 260/280 nm and the final preparation were stored at -20ºC.

FOXP3 genotyping

Genotyping of *FOXP3* g.10403A>G (rs2232365) and g.8048A>C (rs3761548) genetic polymorphisms were assessed by polymerase chain reaction (PCR) followed by enzyme digestion with restriction endonucleases.

For g.10403A>G and g.8048A>C genotyping were used specific oligonucleotide primers (Table 1) according to Paradowska-Gorycka, Jurkowska et al. (2015) and He, Bo et al. (2013), respectively. The polymerase chain reaction (PCR) was conducted using the following conditions: 1X of PCR Buffer (20mM of Tris-HCl ph 8.5; 50m.M of KCl), 0.8 mM of $MgCl_2$, 0.1 mM of dNTP, 0.2μM of each primer, 1.25U of Taq DNA polymerase, 100 ng of genomic

DNA and ultra-pure H2O (Milli-Q) to complete a final volume of 25 μL. Negative controls were employed to make sure that no contaminants were introduced. The cycling protocol used to both *FOXP3* polymorphisms was a denaturation at 94ºC for 5 min, 35 cycles of 45 sec at 94ºC, 45 sec at 59ºC to g.10403A>G or 65ºC to g.8048A>C and 45 sec at 72ºC, and 10 min of final elongation at 72ºC. PCR products (5 μl) of g.10403A>G were digested overnight at 55ºC with 1 unit/reaction of BsmBI restriction endonuclease (New England Biolabs, Beverly, Massachusetts, USA), and the PCR products (6 μl) of g.8048A>C were digested overnight at 37ºC with 2 units/reaction of PstI restriction endonuclease (New England Biolabs, Beverly, Massachusetts, USA). All PCR and digested products were analyzed on polyacrylamide gel (10%), stained with silver nitrate. Specific primers, PCR products and restriction fragments are displayed in Table 1.

Table 2. Oligonucleotides primers sequences, fragment sizes and restriction products of g.10403A>G and g8048A>C genetic polymorphisms

	Oligonucleotide primers	PCR Products	Restriction enzyme	Restriction Products
g.10403A>G	F: 5' - AGGAGAAGGAGTGGGCATTT – 3' R: 5' - TGTGAGTGGAGGAGCTGAGG – 3'	249bp	BsmBI	Allele A: 249bp Alelle G: 132, 117bp
g.8048A>C	F: 5' - GGCAGAGTTGAAATCCAAGC – 3' R: 5' - CAACGTGTGAGAAGGCAGAA – 3'	155bp	PstI	Allele A: 155bp Allele C: 80, 75bp

F: Forward primer; R: Reverse primer

Haplotype analysis

FOXP3 haplotypes were determined based on the genotypes of all study participants using PHASE software version 2.1.1 (Stephens, Smith et al. 2001; Stephens and Scheet 2005).

RNA Isolation and Reverse Transcriptase Reaction

Total cellular RNA was obtained from 61 samples of breast tumor tissue, using TRIzol LS reagent (Invitrogen, Carlsbad, California, USA) according to manufacturer's instructions and quantified using NanoDrop 2000c Spectrophotometer (Thermo Scientific, Wilmington, Delaware, USA). Reverse transcriptase reaction was performed using 500 ng of RNA, 20 units of cloned Moloney Murine Leukemia Virus Reverse Transcriptase (Invitrogen, Carlsbad, California, USA), and 4 units of Recombinant Ribonuclease Inhibitor RNaseOUT (Invitrogen, Carlsbad, California, USA) under the following conditions: 0.4μM of oligo dT, 40mM of Tris-HCl (pH 8.3), 60mM of KCl, 6.4mM of $MgCl_2$, and 0.2mM of dNTP, at 37ºC for 60 min in a Thermal Cycler.

Real Time PCR for FOXP3

Quantitative real time PCR (qRT-PCR) was performed using Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen, Carlsbad, California, USA) on a Step One Real-Time PCR thermal cycler (Applied Biosystems, Foster City, California, USA). The primers used for amplification of *FOXP3* and *β-actin* are described in Table 2. The thermal cycling conditions were 10 minutes at 95ºC, and 40 cycles of 15 seconds at 95ºC and 1 min at 60ºC, followed by melting curve, which consisted of one cycle of 15 seconds at 95ºC, 1 min at 60ºC and growing increase up to 95ºC (0.3ºC/second) with detection of fluorescence at each temperature increase to confirm the specific amplification. In addition, in all experiments, appropriate negative controls containing no template were subjected to the same procedure to exclude or detect any possible contamination. Relative mRNA expression levels of *FOXP3* were calculated according to the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001) and normalized by the previously characterized housekeeping, *β-actin* gene. A commercial pool of human normal mammary gland RNA (Clontech Laboratories, Mountain View, California, USA) was used as a non-neoplastic sample.

Table 2. Quantitative RT-PCR primers sequences.

Gene	GenBank accession number	Primer	Sequence	Melting (TºC)
FOXP3	NM_014009.3	Forward Reverse	5' – CACCTGGCTGGGAAAATGG - 3' 5' - GAGCCCTTGTCGGATGAT - 3'	86ºC
β-actin	NM_001101	Forward Reverse	5' – GGCTTTATTTGTTTTTTTTGTTTTG - 3' 5' - CACCTTCACCGTTCCAGTTTTT - 3'	73ºC

Statistical analysis

The Kruskal-Wallis test was performed to compare the relative expression of *FOXP3* mRNA between different breast cancer subtypes, using GraphPad Prism 6.0 statistical software for Windows (GraphPad Software, La Jolla, California, USA). The correlation analysis of relative expression of FOXP3 mRNA with clinical outcomes and genetic polymorphisms were performed by Spearman Rho test, using statistic software SPSS version 22.0 (SPSS Inc., Chicago, Illinois, USA). A p value <0.05 was considered statistically significant. For all the calculations the outliers data were removed by GraphPad software.

Results

The age of patients involved in the present study ranged from 31 to 99 years, with an average of 54 (±10.9) years. Most patients showed invasive ductal carcinoma (95.1%). The general median tumor size was 3 cm, among them the TN tumors presented the larger size (3.5 cm). The prognostic parameters in total BC patients and in subtypes are shown in Table 3, but some characteristics were not available.

Table 3. Prognostic parameters in total BC patients and in aggressive subtypes.

Prognostic parameters		**Total sample**	**Luminal B HER2+**	**HER2 enriched**	**Triple negative**
Tumor size (n=59)	≤ 3.0cm	33 (55.9%)	14 (73.7%)	9 (69.2%)	10 (37%)
	> 3.0 cm	26 (44.1%)	5 (26.3%)	4 (30.8%)	17 (63%)
TNM staging (n=59)	I and II	36 (61%)	11 (64.7%)	10 (66.7%)	15 (55.6%)
	III and IV	23 (39%)	6 (35.3%)	5 (33.3%)	12 (44.4%)
Histological grade (n=57)	II	17 (29.8%)	7 (36.8%)	4 (36.4%)	6 (22.2%)
	III	40 (70.2%)	12 (63.2%)	7 (63.6%)	21 (77.8%)
Ki-67 (n=55)	Low	4 (7.3%)	3 (18.8%)	0 (0%)	1 (3.7%)
	Moderate	17 (30.9%)	6 (37.5%)	5 (41.7%)	6 (22.2%)
	High	34 (61.8%)	7 (43.7%)	7 (58.3%)	20 (74.1%)
Lymph nodes commitment (n=58)	No	29 (50%)	9 (50%)	9 (64.3%)	11 (42.3%)
	Yes	29 (50%)	9 (50%)	5 (35.7%)	15 (57.7%)

The predominant genotypes of g.10403A>G and g.8048A>C in total BC sample were AG (44.3%) and CC (49.2%), respectively, and the lesser frequent was the AA genotype to both polymorphisms, with 24.6% in g.10403A>G and 11.5% in g.8048A>C. The frequencies of different *FOXP3* haplotypes for total sample and subtypes of BC were determined and are shown in Table 4. The most frequent haplotype in all groups was the AC and the lesser was the AA.

Table 4. Haplotype frequency of *FOXP3* polymorphisms in total sample and aggressive subtypes of breast cancer.

Haplotype	Total sample	LB	HER2+	TN
AC	56 (45.9%)	16 (42.1%)	12 (40%)	28 (51.9%)
AA	1 (0.8%)	1 (2.6%)	0 (0%)	0 (0%)
GC	26 (21.3%)	8 (21.1%)	6 (20%)	12 (22.2%)
GA	39 (32%)	13 (34.2%)	12 (40%)	14 (25.9%)
Total	122 (100.0%)	38 (100.0%)	30 (100.0%)	54 (100.0%)

LB: Luminal B with HER2 overexpression; HER2+: HER2-enriched; TN: triple-negative

The investigation of *FOXP3* mRNA expression by qRT-PCR in breast tumor tissue and in normal mammary gland showed that LB presented 2.6 fold lower *FOXP3* mRNA expression relative to normal mammary gland. HER2+ and TN subtype presented 1.8 and 1.5 folds higher expression, respectively, in relation to normal mammary gland. Grouping all BC subtypes showed no difference in *FOXP3* mRNA expression in relation to normal mammary gland (1 fold). The difference in *FOXP3* mRNA relative expression among the different subtypes was not significant (p=0.175) (Figure 1). Also no difference was observed when the HER2+ and LB was grouped (p=0.459) or when the TN was grouped with HER2+ and compared to LB (p=0.07).

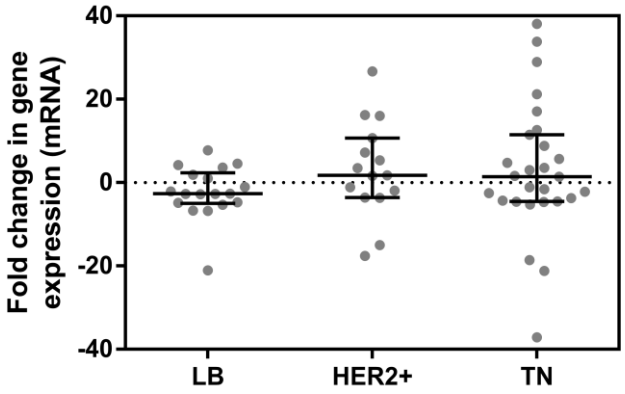

Figure 1. *FOXP3* mRNA relative expression in aggressive breast cancer subtypes. The expression levels of mRNA were calculated according $2^{-\Delta\Delta Ct}$ method, using a normal mammary gland RNA as control. LB: Luminal B HER2+; HER2+: HER2-enriched.; TN: triple-negative.

The correlation analysis between the *FOXP3* mRNA expression and prognostic parameters in total sample and in different subtypes are shown in Table 5. No significant correlations with

prognostic were found. However, in total sample, tendencies of correlation were observed with lower TNM staging (p=0.06; rho=-0.25) and higher histological grade (p=0.08; rho=0.23). In TN subtype also were observed tendencies, such as with lower tumor size (p=0.06; rho=-0.38) and higher lymph nodes commitment (p=0.09; rho=0.34).

Furthermore the *FOXP3* mRNA expression was not correlated with progesterone receptor (p=0.289, rho=-0.14) and HER2 overexpression (0.458; rho=-0.09). However, although not significant, a tendency of negative correlation was observed between *FOXP3* mRNA expression and estrogen receptor status (p=0.07; rho=-0.24).

Table 5. Correlation analysis of *FOXP3* mRNA expression with prognostic parameters in aggressive BC subtypes.

Prognostic parameters	General BC sample	Breast cancer subtypes		
		LB	HER2+	TN
TNM Staging	0.06 (rho=-0.25)	0.240 (rho=-0.30)	0.483 (rho=-0.19)	0.230 (rho=-0.24)
Tumor size	0.724 (rho=-0.05)	0.315 (rho=0.251)	0.592 (rho=-0.16)	0.06 (rho=-0.38)
Ki-67	0.648 (rho=0.06)	0.188 (rho=-0.35)	0.940 (rho=-0.02)	0.590 (rho=0.11)
Histological grade	0.08 (rho=0.23)	0.416 (rho=0.20)	0.418 (rho=-0.24)	0.129 (rho=0.31)
Lymph nodes commitment	0.161 (rho=0.19)	0.524 (rho=-0.16)	0.772 (rho=-0.09)	0.09 (rho=0.34)

LB: Luminal B HER2+; HER2+: HER2-enriched; TN: triple-negative. Spearman rho test; *Value of $p<0.05$ was considered statistically significant.

The *FOXP3* mRNA expression was not different between the genotypes of g.10403A>G polymorphism (p=0.200), and no correlation was found (p=0.08; rho=-0.24). Also was no found significant difference in *FOXP3* expression between the genotypes of g.8048A>C (p=0.232) and neither significant correlation (p=0.191; rho=-0.17) (Figure 2).

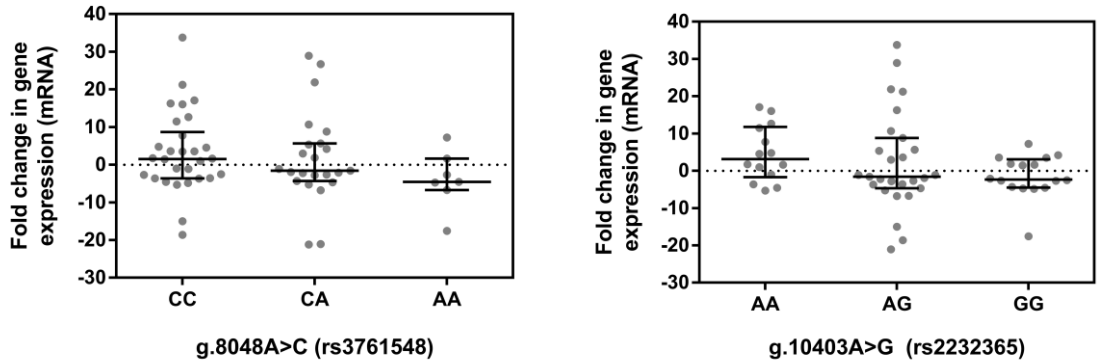

Figure 2. Relative expression of *FOXP3* mRNA in relation to g.8048A>C and g.10403A>G genetic polymorphisms.

Furthermore the *FOXP3* mRNA also was not correlated with haplotypes GC (p=0.244; rho=-0.15) and GA (p=0.791; rho=0.04). However a significant correlation was found between the AC haplotype and *FOXP3* mRNA expression, as shown in Figure 3 (p=0.039; rho=0.27).

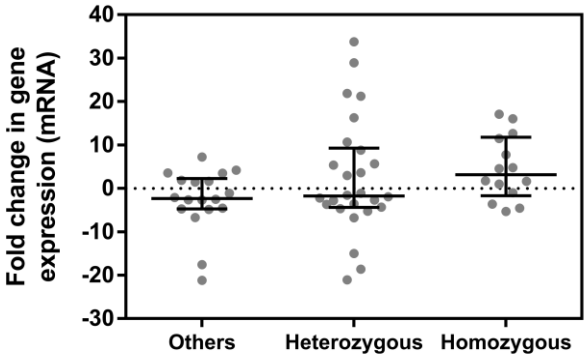

Figure 3. Relative expression of *FOXP3* mRNA in relation to AC haplotype. AC homozygous presented 3.2 folds higher *FOXP3* expression in relation to normal mammary gland, while patients without the AC haplotype presented lesser 2.3 folds expression. A significant correlation was found (p=0.039; rho=0.27). Homozygous: ACAC; Heterozygous: ACGC and ACGA; Others: GCGA; GCGC; GAGA and AAGA.

Furthermore the *FOXP3* expression is significant different between the AC homozygous and GA homozygous (p=0.045). AC homozygous presented 3.2 folds higher *FOXP3* mRNA expression in relation to normal mammary gland, while GA homozygous presented 2.7 folds lesser expression (Figure 4).

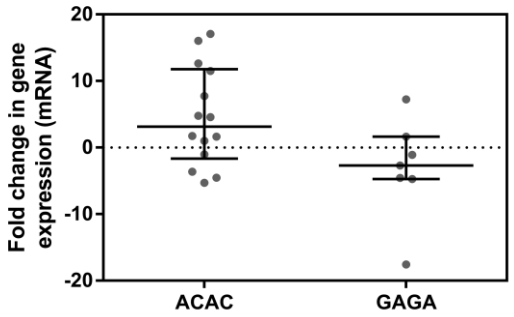

Figure 4. Relative expression of *FOXP3* mRNA in AC and GA homozygous. AC homozygous presented 3.2 folds higher *FOXP3* expression in relation to normal mammary gland, while patients GAGA presented 2.7 folds lesser. A significant difference was found (p=0.045).

Discussion

Many studies have shown that *FOXP3* could be considered a tumor suppressor gene. Particularly in breast cancer, this transcription factor increases p21 expression and inhibits important oncogenes, such as *HER2*, *SKP2* and *c-MYC*, as well the gene of chemokine receptor *CXCR4*, involved in metastatic process of breast tumor, conferring a better prognosis (Douglass, Meeson et al. 2014; Zhang, Xu et al. 2015). A study developed by Recouvreux, Grasso et al. (2016) also showed that FOXP3 interacts with RUNX1, blocking its activity and consequently downregulating *RSPO3* oncogene expression and upregulating *GJA1* tumor suppressor gene expression. Furthermore, FOXP3 also induces the expression of microRNAs, such as miR-146a/b, miR-7 and miR-155, which contribute to the inhibition of breast tumor growth (McInnes, Sadlon et al. 2012; Liu, Liu et al. 2015).

In the present study was found that LB subtype presented 2.6 folds lesser *FOXP3* mRNA expression in relation to normal mammary gland, which is in accordance with literature. It has been reported that in comparison with normal breast epithelial cells, FOXP3 is down-regulated at both transcript and protein levels in BC tissues or cell lines (Zuo, Wang et al. 2007; Douglass, Meeson et al. 2014). Since *FOXP3* has been described as a tumor suppressor gene, its absence may be associated with loss of tumor inhibition.

On the other hand, HER2+ and TN subtypes presented slightly higher *FOXP3* mRNA expression in relation to normal breast, which is in accordance with Ohara, Yamaguchi et al. (2009). These authors suggested that higher *FOXP3* mRNA expression in breast tumor indicates infiltrating Treg cell origin. Studies have shown that Treg infiltration is correlated

with molecular subtypes, of which the luminal A had the lowest infiltration and increased gradually to luminal B, luminal B HER2+, HER2-enriched and the highest, in triple-negative tumors (Liu, Lang et al. 2011; Li, Liu et al. 2013). This difference could be explained by CCL22 expression among molecular subtypes (Li, Liu et al. 2013), since tumor-derived CCL22 possibly promotes Treg recruitment, through its CCR4 receptor (Hirahara, Liu et al. 2006).

FOXP3-expressing Treg cells avoid the anti-tumor activity of immune effector cells in BC microenvironment, resulting in poor prognosis of patients (Ohara, Yamaguchi et al. 2009). Increased Treg infiltration in BC is associated with unfavorable clinicopathological parameters, such as higher Ki-67 and tumor size, worse histologic grade, hormone receptor negativity, HER2 positivity, and tumor recurrence (Bates, Fox et al. 2006; Ladoire, Arnould et al. 2008; Kim, Lee et al. 2014; Lopes, Guembarovski et al. 2014).

In the present study tendencies of correlation between *FOXP3* mRNA expression and some prognostic parameters were found. In the total sample, tendencies were found with estrogen receptor negativity and higher histological grade. Ohara, Yamaguchi et al. (2009), which also analyzed *FOXP3* mRNA expression in breast tumor by qRT-PCR, observed a significant correlation with higher histological grade and progesterone receptor negativity. These authors attributed the correlation with worse prognosis to Treg infiltration.

In TN subtype, tendencies were observed with lower tumor size and lymph node commitment. In contrast with our results,Ortiz-Martinez, Gutierrez-Avino et al. (2016) found, in this subtype, significant association with higher tumor size, on the other hand, they found significant association with lymph node status.

The absence of significant correlations and the contradictory tendencies found in the present study may be due the fact that *FOXP3* mRNA expression can be referent to infiltrating Tregs and breast tumor cells. Our work was limited by lack of information about Treg infiltration and future investigations with additional methodologies are required to confirm the results.

As discussed above, the function of FOXP3 in BC microenvironment has been widely studied. But the transcriptional regulation of this transcription factor itself has not been fully understood, particularly in epithelial cell. So far, cancer research has mostly focused on mutations that alter protein-coding sequences. However, this coding fraction only represents less than 2% of human genome (Weinhold, Jacobsen et al. 2014). Mutations occurring in regulatory regions, depending on whether the binding site of an activating or repressing transcription factor is affected, can result in transcriptional up- or downregulation. If

oncogene or tumor suppressor genes are affected, mutations in regulatory elements may constitute causative events in tumorigenesis (Diederichs, Bartsch et al. 2016).

In the present study two *FOXP3* polymorphisms, g.10403A>G and g.8048A>C , located in intron-1, were analyzed in BC patients. Conserved non-coding sequences (CNS), in intronic regions, are capable to bind transcriptional factors and actuate in concert with *FOXP3* promoter (Zheng, Josefowicz et al. 2010; Maruyama, Konkel et al. 2011).

Our results demonstrate that although *FOXP3* mRNA expression was not correlated with g.10403A>G and g.8048A>C polymorphisms, a significant correlation with AC haplotype was found. AC homozygous presented 3.2 folds higher *FOXP3* mRNA expression in relation to normal mammary gland, while GA homozygous presented 2.7 folds lesser expression. According a work developed by Shen, Chen et al. (2010) psoriatic patients with AA genotype of g.8048A>C polymorphism have reduced *FOXP3* gene expression. These authors demonstrated that the C to A change causes bindings loss to E47 and c-Myb transcription factors, leading to defective transcription of *FOXP3* gene.

Wu, You et al. (2012) performed an extensive search for transcriptional factor-binding sites and found that g.10403A>G SNP is located in a putative binding site for the transcription factor GATA-3. More importantly, only when the A allele exists, this transcription factor can bind the promoter region of *FOXP3*. According Wang, Su et al. (2011) defective function of both GATA-3 and FOXP3 itself led to ablation of Treg cells, suggesting that the combined function of GATA-3 and FOXP3 is essential for FOXP3 expression. In this context, *FOXP3* haplotype with A allele of g.10403A>G and C allele of g.8048C>A may be associated with increased FOXP3 expression, which is in accordance with our results.

Although more studies with analysis of tumor-infiltrating Treg are needed, the present study shows, for the first time, the effect of g.10403A>G and g.8048A>C haplotypes on *FOXP3* gene expression, highlighting the importance to investigate the haplotypes impact in gene expression besides polymorphisms variants separately. Since FOXP3 expression was previous related with clinical parameters of breast cancer, the AC haplotype of gene might be a candidate for prognostic marker for this disease.

Conflict of Interests

The authors declare that there are no conflicts of interest.

Acknowledgments

The author would like to acknowledge the volunteers who made this study possible. This work was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq 303186/2015-1) and Londrina State University Coordination for Post-Graduation (PROPPG-UEL). The authors wish to express their gratitude to Fundação Araucária do Paraná, Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), and Fundação Araucaria for financial support.

References

Bates, G. J., S. B. Fox, et al. (2006). "Quantification of regulatory T cells enables the identification of high-risk breast cancer patients and those at risk of late relapse." J Clin Oncol **24**(34): 5373-5380.

Coates, A. S., E. P. Winer, et al. (2015). "Tailoring therapies--improving the management of early breast cancer: St Gallen International Expert Consensus on the Primary Therapy of Early Breast Cancer 2015." Ann Oncol **26**(8): 1533-1546.

de la Cruz-Merino, L., A. Barco-Sanchez, et al. (2013). "New insights into the role of the immune microenvironment in breast carcinoma." Clin Dev Immunol **2013**: 785317.

Diederichs, S., L. Bartsch, et al. (2016). "The dark matter of the cancer genome: aberrations in regulatory elements, untranslated regions, splice sites, non-coding RNA and synonymous mutations." EMBO Mol Med **8**(5): 442-457.

Douglass, S., A. P. Meeson, et al. (2014). "Breast cancer metastasis: demonstration that FOXP3 regulates CXCR4 expression and the response to CXCL12." J Pathol **234**(1): 74-85.

Fontenot, J. D., M. A. Gavin, et al. (2003). "Foxp3 programs the development and function of CD4+CD25+ regulatory T cells." Nat Immunol **4**(4): 330-336.

He, Y. Q., Q. Bo, et al. (2013). "FoxP3 genetic variants and risk of non-small cell lung cancer in the Chinese Han population." Gene **531**(2): 422-425.

Hirahara, K., L. Liu, et al. (2006). "The majority of human peripheral blood CD4+CD25highFoxp3+ regulatory T cells bear functional skin-homing receptors." J Immunol **177**(7): 4488-4494.

Hori, S., T. Nomura, et al. (2003). "Control of regulatory T cell development by the transcription factor Foxp3." Science **299**(5609): 1057-1061.

Karanikas, V., M. Speletas, et al. (2008). "Foxp3 expression in human cancer cells." J Transl Med **6**: 19.

Kim, S., A. Lee, et al. (2014). "Zonal difference and prognostic significance of foxp3 regulatory T cell infiltration in breast cancer." J Breast Cancer **17**(1): 8-17.

Ladoire, S., L. Arnould, et al. (2008). "Pathologic complete response to neoadjuvant chemotherapy of breast carcinoma is associated with the disappearance of tumor-infiltrating foxp3+ regulatory T cells." Clin Cancer Res **14**(8): 2413-2420.

Li, Y. Q., F. F. Liu, et al. (2013). "Tumor secretion of CCL22 activates intratumoral Treg infiltration and is independent prognostic predictor of breast cancer." PLoS One **8**(10): e76379.

Liu, F., R. Lang, et al. (2011). "CD8(+) cytotoxic T cell and FOXP3(+) regulatory T cell infiltration in relation to breast cancer survival and molecular subtypes." Breast Cancer Res Treat **130**(2): 645-655.

Liu, R., C. Liu, et al. (2015). "FOXP3 controls an miR-146/NF-kappaB negative feedback loop that inhibits apoptosis in breast cancer cells." Cancer Res **75**(8): 1703-1713.

Livak, K. J. and T. D. Schmittgen (2001). "Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method." Methods **25**(4): 402-408.

Lopes, L. F., R. L. Guembarovski, et al. (2014). "FOXP3 transcription factor: a candidate marker for susceptibility and prognosis in triple negative breast cancer." Biomed Res Int **2014**: 341654.

Maruyama, T., J. E. Konkel, et al. (2011). "The molecular mechanisms of Foxp3 gene regulation." Semin Immunol **23**(6): 418-423.

McInnes, N., T. J. Sadlon, et al. (2012). "FOXP3 and FOXP3-regulated microRNAs suppress SATB1 in breast cancer cells." Oncogene **31**(8): 1045-1054.

Merlo, A., P. Casalini, et al. (2009). "FOXP3 expression and overall survival in breast cancer." J Clin Oncol **27**(11): 1746-1752.

Miyan, M., J. Schmidt-Mende, et al. (2016). "Differential tumor infiltration by T-cells characterizes intrinsic molecular subtypes in breast cancer." J Transl Med **14**(1): 227.

Ohara, M., Y. Yamaguchi, et al. (2009). "Possible involvement of regulatory T cells in tumor onset and progression in primary breast cancer." Cancer Immunol Immunother **58**(3): 441-447.

Ortiz-Martinez, F., F. J. Gutierrez-Avino, et al. (2016). "Association of Notch pathway down-regulation with Triple Negative/Basal-like breast carcinomas and high tumor-infiltrating FOXP3+ Tregs." Exp Mol Pathol **100**(3): 460-468.

Paradowska-Gorycka, A., M. Jurkowska, et al. (2015). "Genetic polymorphisms of Foxp3 in patients with rheumatoid arthritis." J Rheumatol **42**(2): 170-180.

Recouvreux, M. S., E. N. Grasso, et al. (2016). "RUNX1 and FOXP3 interplay regulates expression of breast cancer related genes." Oncotarget **7**(6): 6552-6565.

Shen, Z., L. Chen, et al. (2010). "Intron-1 rs3761548 is related to the defective transcription of Foxp3 in psoriasis through abrogating E47/c-Myb binding." J Cell Mol Med **14**(1-2): 226-241.

Sobin, L. H., M. K. Gospodarowicz, et al. (2009). TNM classification of malignant tumours.

Stephens, M. and P. Scheet (2005). "Accounting for decay of linkage disequilibrium in haplotype inference and missing-data imputation." Am J Hum Genet **76**(3): 449-462.

Stephens, M., N. J. Smith, et al. (2001). "A new statistical method for haplotype reconstruction from population data." Am J Hum Genet **68**(4): 978-989.

TCGA (2012). "Comprehensive molecular portraits of human breast tumours." Nature **490**(7418): 61-70.

Wang, Y., M. A. Su, et al. (2011). "An essential role of the transcription factor GATA-3 for the function of regulatory T cells." Immunity **35**(3): 337-348.

Weinhold, N., A. Jacobsen, et al. (2014). "Genome-wide analysis of noncoding regulatory mutations in cancer." Nat Genet **46**(11): 1160-1165.

Wu, Z., Z. You, et al. (2012). "Association between functional polymorphisms of Foxp3 gene and the occurrence of unexplained recurrent spontaneous abortion in a Chinese Han population." Clin Dev Immunol **2012**: 896458.

Zhang, C., Y. Xu, et al. (2015). "FOXP3 suppresses breast cancer metastasis through downregulation of CD44." Int J Cancer **137**(6): 1279-1290.

Zheng, Y., S. Josefowicz, et al. (2010). "Role of conserved non-coding DNA elements in the Foxp3 gene in regulatory T-cell fate." Nature **463**(7282): 808-812.

Zuo, T., R. Liu, et al. (2007). "FOXP3 is a novel transcriptional repressor for the breast cancer oncogene SKP2." J Clin Invest **117**(12): 3765-3773.

Zuo, T., L. Wang, et al. (2007). "FOXP3 is an X-linked breast cancer suppressor gene and an important repressor of the HER-2/ErbB2 oncogene." Cell **129**(7): 1275-1286.

ARTIGO 4

Tumor cytoplasmic FOXP3 expression is correlated with lymph node commitment in breast cancer

Abstract

Studies have demonstrated that breast tumor cells expressing a transcription factor termed forkhead box P3 (FOXP3), which is believed to be restrictively expressed in regulatory T cells (Treg). This protein appears be a potential prognostic factor for this neoplasia, however the mechanisms are still poorly understood. Epigenetic regulation is crucial for controlling the *FOXP3* expression, including DNA methylation. In this context, the present study analyzed the FOXP3-positive mononuclear infiltrating cells, investigated the methylation percentage of *FOXP3* promoter region, and correlated with its gene expression, as well as performed correlation analyzes with prognostic parameters. Twenty five paired tumor and adjacent normal tissue were obtained, of which two were carcinoma in situ (CIS), 20 invasive ductal carcinomas (CDI) and three benign tumors (BG). The gene expression was performed by quantitative real time PCR, the FOXP3 profile by immunohistochemistry and the methylation profiling by Epitect Methyl qPCR array. The median percentage of *FOXP3* promoter methylation in tumors was 62.4%, and no significant difference between BG, CIS and CDI was found (p=0.96). Also no significant difference between tumoral and normal adjacent tissue was observed (p=0.63). The *FOXP3* promoter methylation was not correlated with both mRNA (p=0.315) and protein expression (cytoplasmic: p=0.736; nucleus: p=0.337), suggesting that other mechanisms may be involved in transcriptional regulation of this protein in breast cancer. Cytoplasmic FOXP3 expression by tumor cell was correlated with lymph node commitment (p=0.01), indicating that the localization of protein expression is determinant in prognosis. Furthermore a tendency between intratumoral FOXP3-positive mononuclear infiltrate and higher tumor histological grade was observed (p=0.068), highlighting the importance to analyze this infiltrate in tumor microenvironment. In this context we suggest that FOXP3 could be a promising prognosis marker in human breast cancer.

Keywords: breast cancer, Treg, FOXP3, methylation, prognosis marker.

Introduction

BC is a heterogeneous and phenotypically complex disease, with several biological subtypes that comprises distinct histological features, clinical outcomes and responses to therapy. Approximately 20% of all breast cancer detected through mammography are ductal carcinoma in situ (DCIS), a pre-invasive form of the disease (Ernster, Ballard-Barbash et al. 2002). However, it has been estimated that 20% to 50% of DCIS tumors would progress to invasive ductal carcinoma (IDC) if left untreated (Sanders, Schuyler et al. 2005).

It has been shown that the immune system must participate in the control and elimination of tumor cells, and the presence of a lymphocytic infiltrate, particularly of regulatory T cells (Treg), in cancer tissue is associated with clinical outcome (Bates, Fox et al. 2006; Schmidt, Bohm et al. 2008; Denkert, Loibl et al. 2010).

Many studies have demonstrated breast tumor cells expressing a Treg transcription factor, termed forkhead box P3 (FOXP3) (Karanikas, Speletas et al. 2008; Won, Kim et al. 2013; Douglass, Meeson et al. 2014; Lopes, Guembarovski et al. 2014)

Recent data suggest that FOXP3 expression in tumor cells could be an independent strong prognostic factor for distant metastases (Merlo, Casalini et al. 2009), but in contrast with these data, *FOXP3* was also recently demonstrated to be a tumor suppressor gene, acting as a transcriptional repressor of *SKP2* and *HER2*, two breast cancer important oncogenes (Zuo, Liu et al. 2007; Zuo, Wang et al. 2007).

Various regulation mechanisms of FOXP3 expression have been proposed to regulatory T cells, such as synergistic action of signals downstream of the T-cell receptor (TCR), co-estimulatory molecules and cytokine receptors (Huehn, Polansky et al. 2009). However the exact regulation mechanisms of FOXP3 expression in tumors cells, particularly in BC, are not well understood.

Epigenetic modifications are important in the normal functioning of the cell, from regulating the dynamic expression of essential genes and associated proteins to repressing those that are un-needed. DNA methylation, histone modifications, and non-coding RNA are 3 distinct epigenetic mechanisms, of which the first is considered the major epigenetic modification in mammalian genomes and is known to have profound effects on gene expression (Handy, Castro et al. 2011).

Several groups have observed that epigenetic regulation is crucial for controlling the expression of *FOXP3* locus (Huehn, Polansky et al. 2009). Sequence analyses have revealed three highly conserved non-coding regions in the *FOXP3* locus, including promoter region,

TGFβ-sensitive element and Treg cell-specific demethylated region (TSDR). All of which have been found to be subject to epigenetic modifications and to be involved in transcription regulation of *FOXP3* (Huehn, Polansky et al. 2009).

Biological functions of FOXP3 in tumor cells and its significance remain contradictory and unclear, thus more studies are necessary to clarify its impact on breast cancer progression. In this context, the present study aimed to analyze the methylation profile of *FOXP3* promoter region, as well as its genic expression in breast tumor cells, in addition to analyze the tumor FOXP3-positive mononuclear infiltrate, correlating with clinical outcomes to better comprise its prognostic impact in breast cancer.

Materials and methods

Ethics aspects and sample characterization

Human Ethics Committee of the State University of Londrina, Paraná, Brazil approved the study (CEP/UEL 189/2013, CAAE 17123113400005231). Patients were informed in detail regarding the research and the consent term was obtained. Were obtained 25 breast tumor tissues and their respectively adjacent tissues, totaling 50 samples, of which 3 were diagnosed as benign (BG), 2 as in situ carcinoma (IS) and 20 as invasive ductal carcinoma (IDC). The samples and data relating to clinical outcomes were kindly provided by Cancer Hospital of Londrina, Londrina, Paraná, Brazil (CHL). Clinical parameters included: tumor size, lymph nodes involvement, Ki-67 proliferation index, histological grade, clinicopathological staging (Tumor/Node/Metastasis classification), which were determined according to the Union of International Control of Cancer classification criteria (Sobin, Gospodarowicz et al. 2009).

Genomic DNA extraction

Genomic DNA was obtained from breast tissues using the QIAamp DNA Mini Kit (Qiagen Company, Hilden, Germany), according to the manufacturer instructions. The DNA was eluted in 50 μL of elution buffer. All samples were quantified by NanoDrop 2000c®Spectrophotometer (Thermo Scientific, Wilmington, Delaware, USA) at a wavelength of 260/280 nm and the final preparation were stored at -20ºC.

Methylation qPCR array

The methylation profile was analyzed using the EpiTect Methyl qPCR Array, following the manufacturer's protocol (Qiagen Company, Hilden, Germany). Briefly, samples were treated

with a simple DNA methylation-sensitive and methylation-dependent restriction enzyme digestion without bisulfite conversion. After digestion, the remaining DNA was quantified by DNA methylation real-time polymerase chain reaction (qPCR) array in an StepOne real-time system (Applied Biosystems). Methylation status was expressed as the percentage of the methylated fraction of input DNA, as determined using the EpiTect Methyl DNA methylation PCR data analysis program (Qiagen, http://www.sabiosciences.com/dna_methylation_data_analysis.php), following the manufacturer's instructions. Differential methylated DNA in the samples was divided into three groups: hypermethylated if the percentage of DNA methylation was higher than the sum of two times the standard deviation and mean of the normal samples, normal-like methylation if the percentage of DNA methylation was in range of two times the standard deviation +/- mean of the normal sample and hypomethylated if percentage of DNA methylation was lower than mean minus two times standard deviation of normal sample (Klajic, Fleischer et al. 2013).

RNA Isolation and Reverse Transcriptase Reaction

Total cellular RNA was obtained from 19 samples of breast tissue, using TRIzol LS reagent (Invitrogen, Carlsbad, California, USA) according to manufacturer's instructions and quantified using NanoDrop 2000c Spectrophotometer (Thermo Scientific Inc., Wilmington, USA). Reverse transcriptase reaction was performed using 500 ng of RNA, 20 units of cloned Moloney Murine Leukemia Virus Reverse Transcriptase (Invitrogen, Carlsbad, California, USA), and 4 units of Recombinant Ribonuclease Inhibitor (RNaseOUT, Invitrogen, Carlsbad, California, USA) under the following conditions: 0.4μM of oligo dT, 40mM of Tris-HCl (pH 8.3), 60mM of KCl, 6.4mM of $MgCl_2$, and 0.2mM of dNTP, at 37ºC for 60 min in a Thermal Cycler. Only samples giving absorbance ratios of 1.9 to 2.0 (260/280) were used in the analysis protocols of gene expression. Before assays mRNA, integrity of RNA samples and DNA contamination was analyzed by conventional PCR for β-actin with specific primers as described by Amarante, De Lucca et al. (2005). PCR conditions were: 94ºC for 1 min followed by 35 cycles of 94ºC for 30 sec, 55ºC for 30 sec, 72ºC for 1 min and finally, 72ºC for 10 min. PCR product was analyzed by polyacrylamide gel electrophoresis stained with 10% silver nitrate (AgNO3). All the RNA samples used presented detectable quantities of β-actin mRNA and acceptable integrity during amplification. No contamination with genomic DNA was verified, since all amplified products presented a fragment correspondent to 353 bp.

Real Time PCR for *FOXP3*

Quantitative real time PCR (qRT-PCR) was performed using Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen, Carlsbad, California, USA) on a Step One Real-Time PCR thermal cycler (Applied Biosystems, Foster City, USA). The primers used for amplification of *FOXP3* and *β-actin* are described in Table 1. The thermal cycling conditions were 10 minutes at 95ºC, and 40 cycles of 15 seconds at 95ºC and 1 min at 60ºC, followed by melting curve, which consisted of one cycle of 15 seconds at 95ºC, 1 min at 60ºC and growing increase up to 95ºC (0.3ºC/second) with detection of fluorescence at each temperature increase to confirm the specific amplification. In addition, in all experiments, appropriate negative controls containing no template were subjected to the same procedure to exclude or detect any possible contamination. Relative mRNA expression levels of FOXP3 were calculated according to the $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen 2001) and normalized by the previously characterized house-keeping, *β-actin* gene. A commercial pool of human normal mammary gland RNA (Clontech Laboratories Inc., Mountain View, California, USA) was used as a non-neoplastic sample.

Table 1. Quantitative RT-PCR conditions and primers sequences.

Gene	GenBank accession number	Primer	Sequence	Melting (TºC)
FOXP3	NM_014009.3	Forward Reverse	5' – CACCTGGCTGGGAAAATGG - 3' 5' - GAGCCCTTGTCGGATGAT - 3'	86ºC
β-actin	NM_001101	Forward Reverse	5' – GGCTTTATTTGTTTTTTTTGTTTTG - 3' 5' - CACCTTCACCGTTCCAGTTTTT - 3'	73ºC

Immunohistochemical Staining

Tissue sections of 3 μm were obtained from paraffinized breast tumors samples. Samples were heated at 56°C, deparaffinized in xylene and rehydrated in a graded alcohol series. Antigen retrieval was performed with 8.2 mM citrate and 1.8mM citric acid buffer. Sections were incubated with mouse monoclonal antibody for FOXP3 clone 236A/E7 (eBioscience, San Diego, California, USA) at dilution 1:50, overnight at 4ºC in a humidity chamber. The sections were stabilized at room temperature for 30 min and washed with PBS (phosphate buffered saline) and anti-mouse/rabbit horseradish peroxidase (HRP) secondary antibody (Bio SB Inc. Santa Barbara, California, USA) was applied, followed by diaminobenzidine (DAB) chromogen system (Sigma-Aldrich, USA). Counter staining was performed with Gill's hematoxylin, and slides were set up with coverslips using Canada balsam. FOXP3 was assessed in tumor and adjacent normal tissue. The reading was performed under a light

microscope (Eclipse-E200, Nikon, Japan) by pathologists. It was adopted the German semiquantitative scoring system, considering the immunohistochemical staining intensity and area extent, which has been widely accepted and used in previous studies from Kok, Lee et al. (2010). For every section was given a score according to the intensity of the staining: weak staining = 1, moderate staining = 2, and strong staining = 3. Controls were performed to verify the specificity of primary antibody and all analyses were independently made by at least two pathologists. However, if there was a discrepancy in individual scores, both pathologists reevaluated the immunohistochemical sections by reaching a consensus agreement.

Statistical analysis

The comparison of methylation profile between BG, IS and IDC groups was performed by Kruskall-Wallis test, and between tumor and adjacent normal tissue was performed by Wilcoxon test, using the GraphPad Prism 6.0 statistical software for Windows (GraphPad Software, La Jolla, California, USA). Correlation analyses involving prognostic parameters and percentage of methylation or *FOXP3* mRNA expression were performed by Spearman rho test, while the correlation analyses between prognostic parameters and FOXP3 protein expression were performed by Kendall-s tau-b, using statistic software SPSS version 22.0 (SPSS Inc., Chicago, Illinois, USA). A p value <0.05 was considered statistically significant.

Results

The age of patients ranged from 31 to 87 years, with a median of 58.5 years. The median tumor size was 2.4 cm in invasive ductal carcinoma and 0.7 cm in situ tumor.

The median percentage of *FOXP3* promoter methylation in benign, in situ and invasive ductal carcinoma was 63.51%, 61.33% and 62.56%, respectively. No significant differences were found between these groups (p=0.963). Furthermore, according to Wilcoxon test, also no significant difference was found in *FOXP3* promoter methylation between the tumor and normal adjacent tissues (p=0.633) (Figure 1).

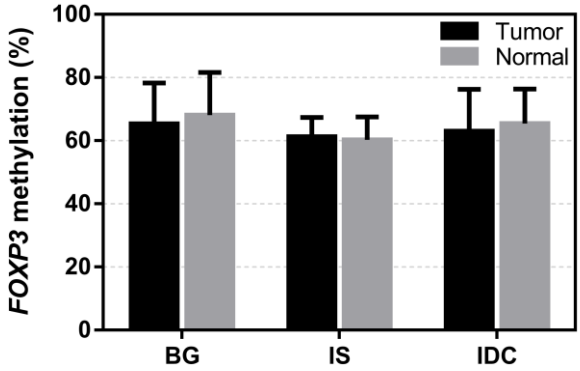

Figure 1. *FOXP3* promoter methylation assay. Percentage of *FOXP3* promoter methylation in tumor and adjacent normal tissues. No significant difference in profile methylation was found between tumor and adjacent normal tissue and neither between benign (BG), in situ (IS) and invasive ductal carcinoma (IDC).

The normal methylation profile was considered between 43.4% and 87.3%, sample with lesser than 43.4% was considered hypometylated and higher than 87.3% was considered hypermethylated. The majority (92%) of samples presented normal methylation, while only one IDC sample presented hypomethylation and one hypermethylation. The *FOXP3* promoter methylation was not correlated with both mRNA expression (p=0.315; r=-0.25) and protein expression, in cytoplasm (p=0.736; r=-0.07) or nucleus (p=0.337; r=0.21).

Although *FOXP3* mRNA expression was not significantly correlated with protein expression (cytoplasm: p=0.355; r=0.23; nucleus: p=0.335; r=0.24), samples with weak staining presented lesser mRNA expression compared with samples with moderated FOXP3 staining, both in nucleus and cytoplasm. Additionally the mRNA expression was not correlated with intra- or peri-tumoral FOXP3-positive mononuclear infiltrate (p=0.801; r=0.07 and p=0.440; r=-0.21, respectively).

No correlation was found between the prognostic parameters and both *FOXP3* promoter methylation and mRNA expression (Table 2).

Table 2. Correlation analysis of prognostic parameters with *FOXP3* promoter methylation and mRNA expression.

Prognostic parameter	Promoter Methylation	mRNA expression
TNM Staging	p=0.843 (r=-0.05)	p=0.397 (r=0.22)
Tumor size	p=0.450 (r=0.17)	p=0.774 (r=0.08)
Ki-67	p=0.225 (r=0.28)	p=0.525 (r=-0.18)
Histological grade	p=0.225 (r=0.27)	p=0.403 (r=-0.22)
LP commitment	p=0.491 (r=-0.16)	p=0.631 (r=-0.13)

Spearman test; LP: lymph node.

Only 12.5% of samples showed nuclear FOXP3 staining. Between the samples that presented nuclear expression, 85.7% had weak and 14.3% moderate staining. None cytoplasmic FOXP3 protein expression was observed in 4.2% of samples. Between the samples that presented cytoplasmic expression, 43.5% presented weak, 52.2% moderate and 4.3% strong staining. The intratumoral FOXP3-positive mononuclear infiltrate was observed in 23.8% of samples, while peritumoral infiltration was observed in 60% of samples (Figure 1).

The correlation analyses between FOXP3 protein expression by tumor and FOXP3-positive mononuclear infiltrating cells with prognostic parameters are listed in Table 3. A significant positive correlation was found between the cytoplasmic FOXP3 staining of tumor cell with lymph node commitment (p=0.01; τ=0.48). None significant correlation was found between the FOXP3-positive mononuclear cell infiltration and prognostic parameters, however was observed a tendency between the intra-tumoral infiltration and higher tumor histological grade (p=0.068; τ=0.30).

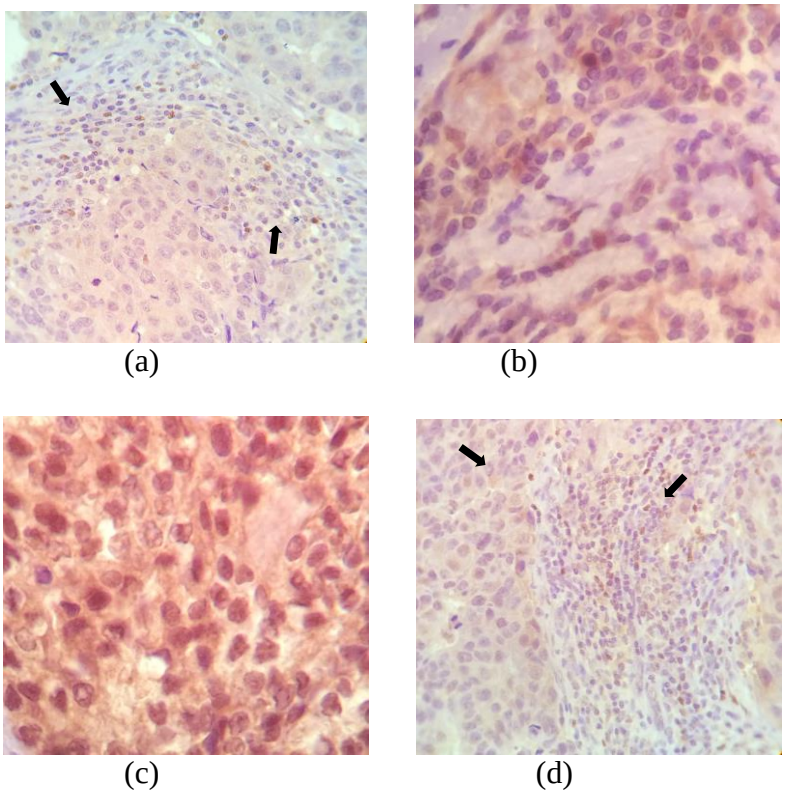

Figure 2. FOXP3 protein expression in breast tumor tissue samples. (a) cytoplasmic expression with weak staining, (b) cytoplasmic expression with moderate staining (c) cytoplasmic and nuclear expression with strong staining (d) intratumoral mononuclear infiltrating cells in breast tumor. The arrows indicated some FOXP3-positive mononuclear cells. Magnification 400x.

Table 3. Correlation analysis of FOXP3 protein expression by tumor and FOXP3-positive mononuclear infiltrate cells with prognostic parameters.

Prognostic parameter	**Tumor expression**		**FOXP3+ mononuclear cell**	
	Cytoplasm	**Nucleus**	**Intratumoral**	**Peritumoral**
TNM Staging	p=0.455 (τ=0.14)	p=0.561 (τ=0.14)	p=0.569 (τ=-0.09)	p=0.665 (τ=0.08)
Tumor size	p=0.242 (τ=-0.19)	p=0.202 (τ=-0.24)	p=0.855 (τ=-0.03)	p=0.738 (τ=0.08)
Ki-67	p=0.916 (τ=-0.03)	p=0.161 (τ=0.32)	p=0.226 (τ=0.22)	p=0.429 (τ=0.19)
Histological grade	p=0.270 (τ=-0.21)	p=0.854 (τ=0.03)	p=0.068 (τ=0.30)	p=0.936 (τ=-0.02)
LP commitment	p=0.01 (τ=0.48)*	p=0.439 (τ=0.18)	p=0.424 (τ=0.16)	p=0.679 (τ=0.09)

Kendall's Tau test; *Value of $p<0.05$ was considered statistically significant. LP: lymph node.

Discussion

Data accumulated over the last decades show that epigenetic changes are the interface between genes and environment, and methylation of suppressor genes in epithelial cells is observed in patients with cancer of different genesis (Feil and Fraga 2011; Kilvitis, Alvarez et al. 2014). DNA methylation is an important regulator of gene transcription, and its role in carcinogenesis has been a topic of considerable interest in the last few years. It is currently well known that cancer is characterized by epigenetic aberrations, and hence it could be postulated that it could have an influence on genes that controls tumor immune response (Ganapathi, Beggs et al. 2014). Hence the DNA methylation status was analyzed in our samples in order to correlate it with the expression levels and clinical parameters of breast cancer.

No significant differences were found between these groups. To date, epigenetic modifications in promotor region of *FOXP3* gene in breast tumor cells remain unstudied yet. Also no significant difference was found in *FOXP3* promoter methylation between the tumor and adjacent healthy tissues in benign, IS and IDC. In this context, Dang, Chen et al. (2014) also found no significant difference in overall methylation of *FOXP3* between hepatocarcinoma and normal liver tissues. However, in human colorectal cancer, Schultze, Andag et al. (2014) found a significant increase of FOXP3 promoter demethylation, and when compared to adjacent healthy tissue, the tumor presented 3.6 folds higher demethylation in *FOXP3* promoter.

The inability of transcription factors to bind to their DNA binding sites when methylated has been proposed as a possible mechanism of gene silencing (Bobetsis, Barros et al. 2007). Studies have shown significant correlations between hypomethylation of *FOXP3* with higher mRNA expression in hepatocellular (Dang, Chen et al. 2014) and human colorectal carcinoma (Ganapathi, Beggs et al. 2014). On the other hand, Lucas, van Baren et al. (2012), which analyzed the methylation status of a conserved region in the first intron of *FOXP3*, found no correlation with mRNA expression in melanoma cells. In the present study the *FOXP3* promoter methylation also was not correlated with both mRNA, cytoplasmic or nuclear protein expression, suggesting that others mechanisms of transcriptional control may be involved, especially since the breast tumor is extremely complex. In addition to DNA methylation, others regulatory transcriptional mechanisms results in aberrant gene expression and are key contributors to breast tumorigenesis, such as histone modifications, non-coding

RNA, specific gene amplifications, deletions, point mutations, chromosome rearrangements and aneuploidy (Dworkin, Huang et al. 2009).

The correlation analysis of clinical parameters and *FOXP3* promoter methylation showed no significant correlation. Buchynska, Iurchenko et al. (2015), studying endometrial cancer, found association of *FOXP3* methylation with tumor differentiation grade and depth of myometrial invasion. The absence of correlation between methylation status and FOXP3 expression together with the small number of samples may explain the lack of correlation with prognostic parameters of BC patients in our study.

Additionally no correlation was found between *FOXP3* mRNA expression and prognostic parameters. Previous studies have shown correlation between *FOXP3* transcript levels with advanced stage (Gupta, Joshi et al. 2007), higher histological grade, higher tumor size and positive lymph-node status in breast cancer (Ohara, Yamaguchi et al. 2009; Ortiz-Martinez, Gutierrez-Avino et al. 2016). These authors associates the *FOXP3* mRNA expression with Treg infiltration, explaining the correlation with poor prognosis, once these cells avoid the anti-tumor activity of immune effector cells in tumor tissue. In this context, both *FOXP3* methylation status and mRNA expression by FOXP3-positive mononuclear cells cannot be ignored, mainly because the intra- and peritumoral infiltrate was present in 23.8% and 60% of samples, respectively. The presence of FOXP3-positive mononuclear cells infiltration in tumor may also explain the lack of correlation of *FOXP3* mRNA with prognostic parameters, since FOXP3 expressed by breast epithelial and regulatory T cells shows an opposite influence in the tumor microenvironment. This implies that to analyze the *FOXP3* methylation status and mRNA expression by breast tumor cells the ideal would be require that tumor-infiltrating T cells be separated from malignant cells.

A significant positive correlation was found between FOXP3 staining in tumor cell cytoplasm and lymph node commitment. Similar result was found by Merlo, Casalini et al. (2009), whose work also showed predominantly cytoplasmic FOXP3 expression by breast tumor cells, which was a strong prognostic factor for lymph node status. According to a study developed by Douglass, Meeson et al. (2014), invasive breast tumor lineages also present FOXP3 located predominantly within cytoplasm. Furthermore they showed that knockdown of FOXP3 in normal human breast epithelial cells with small interfering RNA (siRNA) significantly increased CXCR4, a chemokine receptor that regulates the development of breast cancer by stimulating cell migration towards CXCL12-expressing sites of metastatic spread.

Additionally Zhang, Xu et al. (2015) demonstrated that FOXP3, in nucleus, binds to the promoter of CD44, an adhesion molecule, and inhibits its protein expression, thereby suppressing adhesion and invasion of human breast cancer cells. In this way, FOXP3 enriched in the cytoplasmic fraction of tumor cells is unable to exercise its transcriptional activity, favoring the CXCR4 and CD44 expression and consequently the lymph node and distant organs metastasis. The predominant cytoplasmic expression of FOXP3 in tumor cells may reflect a failure of nuclear translocation, potentially a consequence of cancer-associated mutations (Wang, Liu et al. 2009). In this context, the localization of FOXP3 in tumor cell may be useful in determining the BC prognosis.

Although no significant correlation was found between FOXP3-positive mononuclear infiltrating cells and prognostic parameters, was observed a tendency with higher tumor histological grade. Liu, Lang et al. (2011) found association of intratumoral Treg infiltration with higher histological grade and others unfavorable features, like decreased overall survival and progression-free survival. In a previous study developed by our group research was found a significant correlation between intratumoral FOXP3-positive infiltrate and tumor size in triple-negative subtype (Lopes, Guembarovski et al. 2014).

The prognostic value of Treg infiltrate seems to depend significantly on their location, but possibly it is also dependent on molecular subtype, suggesting that the biologic properties of Treg are influenced by the tumor microenvironment in which they reside (deLeeuw, Kost et al. 2012). Perhaps in the present study the absence of significant correlations of FOXP3-positive mononuclear infiltrate with prognosis may be due to the small sample size and because our sample involve general breast cancer and not a specific subtype.

The correlation of cytoplasmic FOXP3 expression in tumor cell with lymph node commitment and the tendency between FOXP3-positive infiltrate and higher histological grade, suggest its potential as a prognosis marker, which will depend on its location. Knowledge of how this transcription factor is regulated in the tumor cell as well its function may assist in a better understood of breast cancer molecular biology and development of new therapeutic strategies in a personalized medicine.

Conflict of Interests

The authors declare that there are no conflicts of interests.

Acknowledgments

The author would like to acknowledge the volunteers who made this study possible. This work was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Londrina State University Coordination for Post-Graduation (PROPPG-UEL). The authors wish to express their gratitude to Fundação Araucária do Paraná, Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), and Fundação Araucária do Paraná for financial support.

References

Amarante, M. K., F. L. De Lucca, et al. (2005). "Expression of noncoding mRNA in human blood cells activated with synthetic peptide of HIV." Blood Cells Mol Dis **35**(2): 286-290.

Bates, G. J., S. B. Fox, et al. (2006). "Quantification of regulatory T cells enables the identification of high-risk breast cancer patients and those at risk of late relapse." J Clin Oncol **24**(34): 5373-5380.

Bobetsis, Y. A., S. P. Barros, et al. (2007). "Bacterial infection promotes DNA hypermethylation." J Dent Res **86**(2): 169-174.

Buchynska, L. G., N. P. Iurchenko, et al. (2015). "FOXP3 gene promoter methylation in endometrial cancer cells." Exp Oncol **37**(4): 246-249.

Dang, S., P. Chen, et al. (2014). "[Expression and methylation status of Foxp3 in human hepatocellular carcinoma]." Zhonghua Gan Zang Bing Za Zhi **22**(8): 616-619.

deLeeuw, R. J., S. E. Kost, et al. (2012). "The prognostic value of FoxP3+ tumor-infiltrating lymphocytes in cancer: a critical review of the literature." Clinical Cancer Research **18**(11): 3022-3029.

Denkert, C., S. Loibl, et al. (2010). "Tumor-associated lymphocytes as an independent predictor of response to neoadjuvant chemotherapy in breast cancer." J Clin Oncol **28**(1): 105-113.

Douglass, S., A. P. Meeson, et al. (2014). "Breast cancer metastasis: demonstration that FOXP3 regulates CXCR4 expression and the response to CXCL12." J Pathol **234**(1): 74-85.

Dworkin, A. M., T. H. Huang, et al. (2009). "Epigenetic alterations in the breast: Implications for breast cancer detection, prognosis and treatment." Semin Cancer Biol **19**(3): 165-171.

Ernster, V. L., R. Ballard-Barbash, et al. (2002). "Detection of ductal carcinoma in situ in women undergoing screening mammography." J Natl Cancer Inst **94**(20): 1546-1554.

Feil, R. and M. F. Fraga (2011). "Epigenetics and the environment: emerging patterns and implications." Nat Rev Genet **13**(2): 97-109.

Ganapathi, S. K., A. D. Beggs, et al. (2014). "Expression and DNA methylation of TNF, IFNG and FOXP3 in colorectal cancer and their prognostic significance." Br J Cancer **111**(8): 1581-1589.

Gupta, S., K. Joshi, et al. (2007). "Intratumoral FOXP3 expression in infiltrating breast carcinoma: Its association with clinicopathologic parameters and angiogenesis." Acta Oncol **46**(6): 792-797.

Handy, D. E., R. Castro, et al. (2011). "Epigenetic modifications: basic mechanisms and role in cardiovascular disease." Circulation **123**(19): 2145-2156.

Huehn, J., J. K. Polansky, et al. (2009). "Epigenetic control of FOXP3 expression: the key to a stable regulatory T-cell lineage?" Nat Rev Immunol **9**(2): 83-89.

Karanikas, V., M. Speletas, et al. (2008). "Foxp3 expression in human cancer cells." J Transl Med **6**: 19.

Kilvitis, H. J., M. Alvarez, et al. (2014). "Ecological epigenetics." Adv Exp Med Biol **781**: 191-210.

Klajic, J., T. Fleischer, et al. (2013). "Quantitative DNA methylation analyses reveal stage dependent DNA methylation and association to clinico-pathological factors in breast tumors." BMC Cancer **13**: 456.

Kok, L. F., M. Y. Lee, et al. (2010). "Comparing the scoring mechanisms of p16INK4a immunohistochemistry based on independent nucleic stains and independent cytoplasmic stains in distinguishing between endocervical and endometrial adenocarcinomas in a tissue microarray study." Arch Gynecol Obstet **281**(2): 293-300.

Liu, F., R. Lang, et al. (2011). "CD8(+) cytotoxic T cell and FOXP3(+) regulatory T cell infiltration in relation to breast cancer survival and molecular subtypes." Breast Cancer Res Treat **130**(2): 645-655.

Livak, K. J. and T. D. Schmittgen (2001). "Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method." Methods **25**(4): 402-408.

Lopes, L. F., R. L. Guembarovski, et al. (2014). "FOXP3 transcription factor: a candidate marker for susceptibility and prognosis in triple negative breast cancer." Biomed Res Int **2014**: 341654.

Lucas, S., N. van Baren, et al. (2012). "Demethylation of the FOXP3 gene in human melanoma cells precludes the use of this epigenetic mark for quantification of Tregs in unseparated melanoma samples." Int J Cancer **130**(8): 1960-1966.

Merlo, A., P. Casalini, et al. (2009). "FOXP3 expression and overall survival in breast cancer." J Clin Oncol **27**(11): 1746-1752.

Ohara, M., Y. Yamaguchi, et al. (2009). "Possible involvement of regulatory T cells in tumor onset and progression in primary breast cancer." Cancer Immunol Immunother **58**(3): 441-447.

Ortiz-Martinez, F., F. J. Gutierrez-Avino, et al. (2016). "Association of Notch pathway down-regulation with Triple Negative/Basal-like breast carcinomas and high tumor-infiltrating FOXP3+ Tregs." Exp Mol Pathol **100**(3): 460-468.

Sanders, M. E., P. A. Schuyler, et al. (2005). "The natural history of low-grade ductal carcinoma in situ of the breast in women treated by biopsy only revealed over 30 years of long-term follow-up." Cancer **103**(12): 2481-2484.

Schmidt, M., D. Bohm, et al. (2008). "The humoral immune system has a key prognostic impact in node-negative breast cancer." Cancer Res **68**(13): 5405-5413.

Schultze, F. C., R. Andag, et al. (2014). "FoxP3 demethylation is increased in human colorectal cancer and rat cholangiocarcinoma tissue." Clin Biochem **47**(3): 201-205.

Sobin, L. H., M. K. Gospodarowicz, et al. (2009). TNM classification of malignant tumours.

Wang, L., R. Liu, et al. (2009). "Somatic single hits inactivate the X-linked tumor suppressor FOXP3 in the prostate." Cancer Cell **16**(4): 336-346.

Won, K. Y., H. S. Kim, et al. (2013). "Tumoral FOXP3 has potential oncogenic function in conjunction with the p53 tumor suppressor protein and infiltrated Tregs in human breast carcinomas." Pathol Res Pract **209**(12): 767-773.

Zhang, C., Y. Xu, et al. (2015). "FOXP3 suppresses breast cancer metastasis through downregulation of CD44." Int J Cancer **137**(6): 1279-1290.

Zuo, T., R. Liu, et al. (2007). "FOXP3 is a novel transcriptional repressor for the breast cancer oncogene SKP2." J Clin Invest **117**(12): 3765-3773.

Zuo, T., L. Wang, et al. (2007). "FOXP3 is an X-linked breast cancer suppressor gene and an important repressor of the HER-2/ErbB2 oncogene." Cell **129**(7): 1275-1286.

5. CONCLUSÃO

- ✓ Foi encontrada uma associação significativa do genótipo AA do polimorfismo g.10403A>G com risco do câncer de mama. Enquanto o genótipo GG foi correlacionado a pior prognóstico, sendo maior índice de proliferação celular Ki-67 no subtipo HER2-superexpresso e estadiamento mais avançado no subtipo triplo-negativo.

- ✓ O genótipo AA do polimorfismo g.8048A>C mostrou correlação com maior Ki-67 e menor grau histológico, no subtipo HER2-superexpresso.

- ✓ Embora não tenha sido encontrada diferença significativa na distribuição de haplótipos entre controles e pacientes com câncer de mama, a análise com parâmetros clínicos revelou correlação significativa do haplótipo GA com menor grau histológico e maior Ki-67 no subtipo HER2-superexpresso e maior estadimento em triplo-negativo. Por outro lado o haplótipo AC foi correlacionado com menor Ki-67 e estadiamento nos subtipos HER2-superexpresso e triplo-negativo, respectivamente.

- ✓ O haplótipo AC foi correlacionado com maior expressão de RNAm de *FOXP3*. Pacientes homozigotos para este haplótipo apresentam 3,2 vezes maior expressão de RNAm de *FOXP3*.

- ✓ Os subtipos de câncer de mama analisados neste trabalho mostraram diferentes níveis de expressão de RNAm de *FOXP3*. O subtipo Luminal B HER2+ apresentou 2,6 vezes menos expressão de *FOXP3* comparado à glândula mamária normal, enquanto a expressão em triplo-negativo (1,5 vezes) e HER2-superexpresso (1,8 vezes) foi ligeiramente maior que o controle de mama normal.

- ✓ A expressão de RNAm de *FOXP3* não foi correlacionada com os parâmetros prognósticos das pacientes com câncer de mama.

- ✓ A porcentagem de metilação da região promotora de *FOXP3* não foi significativamente diferente entre os tumores benignos, carcinomas ductais *in situ* e ductais invasivos. Também não foi encontrada diferença entre o tecido tumoral e

normal adjacente.

- ✓ A metilação da região promotora não foi correlacionada com a expressão de RNAm e proteica de FOXP3, bem como com parâmetros prognósticos das pacientes.

- ✓ A expressão proteica citoplasmática de FOXP3 em células tumorais de mama foi correlacionada com maior comprometimento de linfonodos. Além disso, foi observada uma tendência de correlação entre o infiltrado de células mononucleares FOXP3-positivas com maior grau histológico tumoral.

6. REFERÊNCIAS

AMARANTE, M. K. et al. Expression of noncoding mRNA in human blood cells activated with synthetic peptide of HIV. **Blood Cells Mol Dis,** v. 35, n. 2, p. 286-90, Sep-Oct 2005.

BENNETT, C. L. et al. A rare polyadenylation signal mutation of the FOXP3 gene (AAUAAA-->AAUGAA) leads to the IPEX syndrome. **Immunogenetics,** v. 53, n. 6, p. 435-9, Aug 2001.

BETTELLI, E.; DASTRANGE, M.; OUKKA, M. Foxp3 interacts with nuclear factor of activated T cells and NF-kappa B to repress cytokine gene expression and effector functions of T helper cells. **Proc Natl Acad Sci U S A,** v. 102, n. 14, p. 5138-43, Apr 5 2005.

BOSCH, A. et al. Triple-negative breast cancer: molecular features, pathogenesis, treatment and current lines of research. **Cancer Treat Rev,** v. 36, n. 3, p. 206-15, May 2010.

BRASIL, Ministério da Saúde. Sistema de Informações sobre Mortalidade. 2016. Disponível em: < http://svs.aids.gov.br/dashboard/situacao/saude.show.mtw>. Acesso em: 24 setembro 2016.

BRUNKOW, M. E. et al. Disruption of a new forkhead/winged-helix protein, scurfin, results in the fatal lymphoproliferative disorder of the scurfy mouse. **Nat Genet,** v. 27, n. 1, p. 68-73, Jan 2001.

CAROTENUTO, P. et al. Triple negative breast cancer: from molecular portrait to therapeutic intervention. **Crit Rev Eukaryot Gene Expr,** v. 20, n. 1, p. 17-34, 2010.

CHEANG, M. C. et al. Ki67 index, HER2 status, and prognosis of patients with luminal B breast cancer. **J Natl Cancer Inst,** v. 101, n. 10, p. 736-50, May 20 2009.

CHEN, Y. et al. DNA binding by FOXP3 domain-swapped dimer suggests mechanisms of long-range chromosomal interactions. **Nucleic Acids Res,** v. 43, n. 2, p. 1268-82, Jan 2015.

CITRI, A.; SKARIA, K. B.; YARDEN, Y. The deaf and the dumb: the biology of ErbB-2 and ErbB-3. **Exp Cell Res,** v. 284, n. 1, p. 54-65, Mar 10 2003.

CURIEL, T. J. Regulatory T cells and treatment of cancer. **Curr Opin Immunol,** v. 20, n. 2, p. 241-6, Apr 2008.

DAWSON, S. J.; PROVENZANO, E.; CALDAS, C. Triple negative breast cancers: clinical and prognostic implications. **Eur J Cancer,** v. 45 Suppl 1, p. 27-40, Sep 2009.

DOUGLASS, S. et al. The role of FOXP3 in the development and metastatic spread of breast cancer. **Cancer Metastasis Rev,** v. 31, n. 3-4, p. 843-54, Dec 2012.

DOUGLASS, S. et al. Breast cancer metastasis: demonstration that FOXP3 regulates CXCR4 expression and the response to CXCL12. **J Pathol,** v. 234, n. 1, p. 74-85, Sep 2014.

EHRLICH, M. et al. Amount and distribution of 5-methylcytosine in human DNA from

different types of tissues of cells. **Nucleic Acids Res,** v. 10, n. 8, p. 2709-21, Apr 24 1982.

FLOESS, S. et al. Epigenetic control of the foxp3 locus in regulatory T cells. **PLoS Biol,** v. 5, n. 2, p. e38, Feb 2007.

FONTENOT, JASON D.; GAVIN, MARC A.; RUDENSKY, ALEXANDER Y. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. **Nat Immunol,** v. 4, n. 4, p. 330-336, 2003.

FRATTINI, V. et al. FOXP3, a novel glioblastoma oncosuppressor, affects proliferation and migration. **Oncotarget,** v. 3, n. 10, p. 1146-57, Oct 2012.

FRIDMAN, W. H. et al. The immune contexture in human tumours: impact on clinical outcome. **Nat Rev Cancer,** v. 12, n. 4, p. 298-306, Apr 2012.

GAO, L. et al. Polymorphisms in the FOXP3 gene in Han Chinese psoriasis patients. **J Dermatol Sci,** v. 57, n. 1, p. 51-6, Jan 2010.

GERDES, J. et al. Cell cycle analysis of a cell proliferation-associated human nuclear antigen defined by the monoclonal antibody Ki-67. **J Immunol,** v. 133, n. 4, p. 1710-5, Oct 1984.

GOLDHIRSCH, A. et al. Personalizing the treatment of women with early breast cancer: highlights of the St Gallen International Expert Consensus on the Primary Therapy of Early Breast Cancer 2013. **Ann Oncol,** v. 24, n. 9, p. 2206-23, Sep 2013.

GU-TRANTIEN, C. et al. CD4(+) follicular helper T cell infiltration predicts breast cancer survival. **J Clin Invest,** v. 123, n. 7, p. 2873-92, Jul 2013.

HANAHAN, D.; WEINBERG, R. A. Hallmarks of cancer: the next generation. **Cell,** v. 144, n. 5, p. 646-74, Mar 04 2011.

HAO, Q. et al. FOXP3 inhibits NF-kappaB activity and hence COX2 expression in gastric cancer cells. **Cell Signal,** v. 26, n. 3, p. 564-9, Mar 2014.

HARVEY, J. M. et al. Estrogen receptor status by immunohistochemistry is superior to the ligand-binding assay for predicting response to adjuvant endocrine therapy in breast cancer. **J Clin Oncol,** v. 17, n. 5, p. 1474-81, May 1999.

HASLAM, S. Z. The ontogeny of mouse mammary gland responsiveness to ovarian steroid hormones. **Endocrinology,** v. 125, n. 5, p. 2766-72, Nov 1989.

HE, Y. Q. et al. FoxP3 genetic variants and risk of non-small cell lung cancer in the Chinese Han population. **Gene,** v. 531, n. 2, p. 422-5, Dec 1 2013.

HENDERSON, I. C.; PATEK, A. J. The relationship between prognostic and predictive factors in the management of breast cancer. **Breast Cancer Res Treat,** v. 52, n. 1-3, p. 261-88, 1998.

HINSHELWOOD, R. A.; CLARK, S. J. Breast cancer epigenetics: normal human mammary epithelial cells as a model system. **J Mol Med (Berl),** v. 86, n. 12, p. 1315-28, Dec 2008.

HONDERMARCK, H. et al. Proteomics of breast cancer for marker discovery and signal pathway profiling. **Proteomics,** v. 1, n. 10, p. 1216-32, Oct 2001.

HORI, S.; NOMURA, T.; SAKAGUCHI, S. Control of regulatory T cell development by the transcription factor Foxp3. **Science,** v. 299, n. 5609, p. 1057-61, Feb 14 2003.

HUANG, Y. et al. CD4+ and CD8+ T cells have opposing roles in breast cancer progression and outcome. **Oncotarget,** v. 6, n. 19, p. 17462-78, Jul 10 2015.

HUEHN, J.; POLANSKY, J. K.; HAMANN, A. Epigenetic control of FOXP3 expression: the key to a stable regulatory T-cell lineage? **Nat Rev Immunol,** v. 9, n. 2, p. 83-9, Feb 2009.

INCA. **Estimate 2016: Cancer Incidence in Brazil**. Rio de Janeiro**:** 51 p. 2016.

JAHAN, P. et al. Association of FOXP3 (rs3761548) promoter polymorphism with nondermatomal vitiligo: A study from India. **J Am Acad Dermatol,** v. 69, n. 2, p. 262-6, Aug 2013.

JANSSEN, E. M. et al. CD4+ T cells are required for secondary expansion and memory in CD8+ T lymphocytes. **Nature,** v. 421, n. 6925, p. 852-6, Feb 20 2003.

JIANG, L. L.; RUAN, L. W. Association between FOXP3 promoter polymorphisms and cancer risk: A meta-analysis. **Oncol Lett,** v. 8, n. 6, p. 2795-2799, Dec 2014.

JOHANSSON, I. et al. Molecular profiling of male breast cancer - lost in translation? **Int J Biochem Cell Biol,** v. 53, p. 526-35, Aug 2014.

JONES, P. A.; BAYLIN, S. B. The epigenomics of cancer. **Cell,** v. 128, n. 4, p. 683-92, Feb 23 2007.

KARANIKAS, V. et al. Foxp3 expression in human cancer cells. **J Transl Med,** v. 6, p. 19, 2008.

KENNECKE, H. et al. Metastatic behavior of breast cancer subtypes. **J Clin Oncol,** v. 28, n. 20, p. 3271-7, Jul 10 2010.

KERKAR, S. P.; RESTIFO, N. P. Cellular constituents of immune escape within the tumor microenvironment. **Cancer Res,** v. 72, n. 13, p. 3125-30, Jul 1 2012.

KETTERHAGEN, J. P.; QUACKENBUSH, S. R.; HAUSHALTER, R. A. Tumor histology as a prognostic determinant in carcinoma of the breast. **Surg Gynecol Obstet,** v. 158, n. 2, p. 120-3, Feb 1984.

KIM, H. P.; LEONARD, W. J. CREB/ATF-dependent T cell receptor-induced FoxP3 gene expression: a role for DNA methylation. **J Exp Med,** v. 204, n. 7, p. 1543-51, Jul 9 2007.

KIM, M. H.; KOO, J. S.; LEE, S. FOXP3 expression is related to high Ki-67 index and poor prognosis in lymph node-positive breast cancer patients. **Oncology,** v. 85, n. 2, p. 128-36, 2013.

KLAJIC, J. et al. Quantitative DNA methylation analyses reveal stage dependent DNA methylation and association to clinico-pathological factors in breast tumors. **BMC Cancer,** v. 13, p. 456, Oct 05 2013.

KOK, L. F. et al. Comparing the scoring mechanisms of p16INK4a immunohistochemistry based on independent nucleic stains and independent cytoplasmic stains in distinguishing between endocervical and endometrial adenocarcinomas in a tissue microarray study. **Arch Gynecol Obstet,** v. 281, n. 2, p. 293-300, Feb 2010.

KUREBAYASHI, J. Possible treatment strategies for triple-negative breast cancer on the basis of molecular characteristics. **Breast Cancer,** v. 16, n. 4, p. 275-80, 2009.

LADOIRE, S. et al. Presence of Foxp3 expression in tumor cells predicts better survival in HER2-overexpressing breast cancer patients treated with neoadjuvant chemotherapy. **Breast Cancer Res Treat,** v. 125, n. 1, p. 65-72, Jan 2011.

LEHMANN, B. D. et al. Identification of human triple-negative breast cancer subtypes and preclinical models for selection of targeted therapies. **J Clin Invest,** v. 121, n. 7, p. 2750-67, Jul 2011.

LEONG, A. S.; ZHUANG, Z. The changing role of pathology in breast cancer diagnosis and treatment. **Pathobiology,** v. 78, n. 2, p. 99-114, 2011.

LESTER, S. C. et al. Protocol for the examination of specimens from patients with invasive carcinoma of the breast. **Arch Pathol Lab Med,** v. 133, n. 10, p. 1515-38, Oct 2009.

LI, W. et al. Identification of a tumor suppressor relay between the FOXP3 and the Hippo pathways in breast and prostate cancers. **Cancer Res,** v. 71, n. 6, p. 2162-71, Mar 15 2011.

LIU, R. et al. FOXP3 controls an miR-146/NF-kappaB negative feedback loop that inhibits apoptosis in breast cancer cells. **Cancer Res,** v. 75, n. 8, p. 1703-13, Apr 15 2015.

LIVAK, K. J.; SCHMITTGEN, T. D. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. **Methods,** v. 25, n. 4, p. 402-8, Dec 2001.

LOPES, L. F. et al. FOXP3 transcription factor: a candidate marker for susceptibility and prognosis in triple negative breast cancer. **Biomed Res Int,** v. 2014, p. 341654, 2014.

MAEGAWA, R. O.; TANG, S. C. Triple-negative breast cancer: unique biology and its management. **Cancer Invest,** v. 28, n. 8, p. 878-83, Oct 2010.

MANTEL, P. Y. et al. Molecular mechanisms underlying FOXP3 induction in human T cells. **J Immunol,** v. 176, n. 6, p. 3593-602, Mar 15 2006.

MCINNES, N. et al. FOXP3 and FOXP3-regulated microRNAs suppress SATB1 in breast cancer cells. **Oncogene,** v. 31, n. 8, p. 1045-54, Feb 23 2012.

MERLO, A. et al. FOXP3 expression and overall survival in breast cancer. **J Clin Oncol,** v.

27, n. 11, p. 1746-52, Apr 10 2009.

NAGAR, M. et al. Epigenetic inheritance of DNA methylation limits activation-induced expression of FOXP3 in conventional human CD25-CD4+ T cells. **Int Immunol,** v. 20, n. 8, p. 1041-55, Aug 2008.

ODA, J. M. et al. Genetic polymorphism in FOXP3 gene: imbalance in regulatory T-cell role and development of human diseases. **J Genet,** v. 92, n. 1, p. 163-71, Apr 2013.

PARADOWSKA-GORYCKA, A. et al. Genetic polymorphisms of Foxp3 in patients with rheumatoid arthritis. **J Rheumatol,** v. 42, n. 2, p. 170-80, Feb 2015.

PARKER, J. S. et al. Supervised risk predictor of breast cancer based on intrinsic subtypes. **J Clin Oncol,** v. 27, n. 8, p. 1160-7, Mar 10 2009.

PERERA, F. P. Environment and cancer: who are susceptible? **Science,** v. 278, n. 5340, p. 1068-73, Nov 7 1997.

PEROU, C. M. et al. Molecular portraits of human breast tumours. **Nature,** v. 406, n. 6797, p. 747-52, Aug 17 2000.

PICCART-GEBHART, M. J. et al. Trastuzumab after adjuvant chemotherapy in HER2-positive breast cancer. **N Engl J Med,** v. 353, n. 16, p. 1659-72, Oct 20 2005.

PRAT, A. et al. Molecular characterization of basal-like and non-basal-like triple-negative breast cancer. **Oncologist,** v. 18, n. 2, p. 123-33, 2013.

QUAGLINO, P. et al. FoxP3 expression on melanoma cells is related to early visceral spreading in melanoma patients treated by electrochemotherapy. **Pigment Cell Melanoma Res,** v. 24, n. 4, p. 734-6, Aug 2011.

RECOUVREUX, M. S. et al. RUNX1 and FOXP3 interplay regulates expression of breast cancer related genes. **Oncotarget,** v. 7, n. 6, p. 6552-65, Feb 9 2016.

REDIG, A. J.; MCALLISTER, S. S. Breast cancer as a systemic disease: a view of metastasis. **J Intern Med,** v. 274, n. 2, p. 113-26, Aug 2013.

ROSEN, P. P. Adenomyoepithelioma of the breast. **Hum Pathol,** v. 18, n. 12, p. 1232-7, Dec 1987.

SAHIN, M. et al. DNA methylation or histone modification status in metastasis and angiogenesis-related genes: a new hypothesis on usage of DNMT inhibitors and S-adenosylmethionine for genome stability. **Cancer Metastasis Rev,** v. 29, n. 4, p. 655-76, Dec 2010.

SAKAGUCHI, S. et al. Regulatory T cells and immune tolerance. **Cell,** v. 133, n. 5, p. 775-87, May 30 2008.

SCHREIBER, R. D.; OLD, L. J.; SMYTH, M. J. Cancer immunoediting: integrating immunity's roles in cancer suppression and promotion. **Science,** v. 331, n. 6024, p. 1565-70,

Mar 25 2011.

SHANG, B.; LIU, Y.; JIANG, S. J. Prognostic value of tumor-infiltrating FoxP3+ regulatory T cells in cancers: a systematic review and meta-analysis. **Sci Rep,** v. 5, p. 15179, 2015.

SHEN, Z. et al. Intron-1 rs3761548 is related to the defective transcription of Foxp3 in psoriasis through abrogating E47/c-Myb binding. **J Cell Mol Med,** v. 14, n. 1-2, p. 226-41, Jan 2010.

SMITH, E. L. et al. Splice variants of human FOXP3 are functional inhibitors of human CD4+ T-cell activation. **Immunology,** v. 119, n. 2, p. 203-11, Oct 2006.

SOBIN, L. H.;; GOSPODAROWICZ, M. K.;; WITTEKIND, C. **TNM classification of malignant tumours**. 2009.

SONG, P. et al. Association between FOXP3 polymorphisms and vitiligo in a Han Chinese population. **Br J Dermatol,** v. 169, n. 3, p. 571-8, Sep 2013.

SONG, Q. H. et al. An association study of single nucleotide polymorphisms of the FOXP3 intron-1 and the risk of Psoriasis vulgaris. **Indian J Biochem Biophys,** v. 49, n. 1, p. 25-35, Feb 2012.

SORLIE, T. Molecular portraits of breast cancer: tumour subtypes as distinct disease entities. **Eur J Cancer,** v. 40, n. 18, p. 2667-75, Dec 2004.

SORLIE, T. et al. Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. **Proc Natl Acad Sci U S A,** v. 98, n. 19, p. 10869-74, Sep 11 2001.

STEPHENS, M.; SCHEET, P. Accounting for decay of linkage disequilibrium in haplotype inference and missing-data imputation. **Am J Hum Genet,** v. 76, n. 3, p. 449-62, Mar 2005.

STEPHENS, M.; SMITH, N. J.; DONNELLY, P. A new statistical method for haplotype reconstruction from population data. **Am J Hum Genet,** v. 68, n. 4, p. 978-89, Apr 2001.

TAHERIAN-FARD, A.; SRIHARI, S.; RAGAN, M. A. Breast cancer classification: linking molecular mechanisms to disease prognosis. **Brief Bioinform,** v. 16, n. 3, p. 461-74, May 2015.

TANG, Y. et al. Prognostic significance of KAI1/CD82 in human melanoma and its role in cell migration and invasion through the regulation of ING4. **Carcinogenesis,** v. 35, n. 1, p. 86-95, Jan 2014.

TOKER, A.; HUEHN, J. To be or not to be a Treg cell: lineage decisions controlled by epigenetic mechanisms. **Sci Signal,** v. 4, n. 158, p. pe4, 2011.

TONE, Y. et al. Smad3 and NFAT cooperate to induce Foxp3 expression through its enhancer. **Nat Immunol,** v. 9, n. 2, p. 194-202, Feb 2008.

VARGO-GOGOLA, T.; ROSEN, J. M. Modelling breast cancer: one size does not fit all. **Nat**

Rev Cancer, v. 7, n. 9, p. 659-72, Sep 2007.

VIGNALI, D.A.A.; COLLISON, L.W.; WORKMAN, C.J. How regulatory T cells work. **Nature Immunology,** v. 8, p. 9, 2008.

WAJED, S. A.; LAIRD, P. W.; DEMEESTER, T. R. DNA methylation: an alternative pathway to cancer. **Ann Surg,** v. 234, n. 1, p. 10-20, Jul 2001.

WANG, H. Y. et al. Tumor-specific human CD4+ regulatory T cells and their ligands: implications for immunotherapy. **Immunity,** v. 20, n. 1, p. 107-18, Jan 2004.

WANG, L. et al. Somatic single hits inactivate the X-linked tumor suppressor FOXP3 in the prostate. **Cancer Cell,** v. 16, n. 4, p. 336-46, Oct 6 2009.

WANG, Y.; SU, M. A.; WAN, Y. Y. An essential role of the transcription factor GATA-3 for the function of regulatory T cells. **Immunity,** v. 35, n. 3, p. 337-48, Sep 23 2011.

WEIGELT, B. et al. Breast cancer molecular profiling with single sample predictors: a retrospective analysis. **Lancet Oncol,** v. 11, n. 4, p. 339-49, Apr 2010.

WIDSCHWENDTER, M.; JONES, P. A. The potential prognostic, predictive, and therapeutic values of DNA methylation in cancer. Commentary re: J. Kwong et al., Promoter hypermethylation of multiple genes in nasopharyngeal carcinoma. Clin. Cancer Res., 8: 131-137, 2002, and H-Z. Zou et al., Detection of aberrant p16 methylation in the serum of colorectal cancer patients. Clin. Cancer Res., 8: 188-191, 2002. **Clin Cancer Res,** v. 8, n. 1, p. 17-21, Jan 2002.

WOLF, A. M. et al. Role of forkhead box protein 3 expression in invasive breast cancer. **J Clin Oncol,** v. 25, n. 28, p. 4499-500; author reply 4500-1, Oct 1 2007.

WON, K. Y. et al. Tumoral FOXP3 has potential oncogenic function in conjunction with the p53 tumor suppressor protein and infiltrated Tregs in human breast carcinomas. **Pathol Res Pract,** v. 209, n. 12, p. 767-73, Dec 2013.

WU, Z. et al. Association between functional polymorphisms of Foxp3 gene and the occurrence of unexplained recurrent spontaneous abortion in a Chinese Han population. **Clin Dev Immunol,** v. 2012, p. 896458, 2012.

XUE, L. et al. Expression of FOXP3 in esophageal squamous cell carcinoma relating to the clinical data. **Dis Esophagus,** v. 23, n. 4, p. 340-6, May 2010.

YAMAMOTO, T. et al. Similarity of protein encoded by the human c-erb-B-2 gene to epidermal growth factor receptor. **Nature,** v. 319, n. 6050, p. 230-4, Jan 16-22 1986.

YAQUB, S.; AANDAHL, E. M. Inflammation versus adaptive immunity in cancer pathogenesis. **Crit Rev Oncog,** v. 15, n. 1-2, p. 43-63, 2009.

YE, J.; LIVERGOOD, R. S.; PENG, G. The role and regulation of human Th17 cells in tumor immunity. **Am J Pathol,** v. 182, n. 1, p. 10-20, Jan 2013.

ZHANG, C. et al. FOXP3 suppresses breast cancer metastasis through downregulation of CD44. **Int J Cancer,** v. 137, n. 6, p. 1279-90, Sep 15 2015.

ZHANG, H. Y.; SUN, H. Up-regulation of Foxp3 inhibits cell proliferation, migration and invasion in epithelial ovarian cancer. **Cancer Lett,** v. 287, n. 1, p. 91-7, Jan 1 2010.

ZHENG, Y. et al. Genome-wide analysis of Foxp3 target genes in developing and mature regulatory T cells. **Nature,** v. 445, n. 7130, p. 936-40, Feb 22 2007.

ZIEGLER, S. F. FOXP3: of mice and men. **Annu Rev Immunol,** v. 24, p. 209-26, 2006.

ZUO, T. et al. FOXP3 is a novel transcriptional repressor for the breast cancer oncogene SKP2. **J Clin Invest,** v. 117, n. 12, p. 3765-73, Dec 2007.

ZUO, T. et al. FOXP3 is an X-linked breast cancer suppressor gene and an important repressor of the HER-2/ErbB2 oncogene. **Cell,** v. 129, n. 7, p. 1275-86, Jun 29 2007.

7. ANEXOS

ANEXO A

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

ANEXO B

UNIVERSIDADE
ESTADUAL DE LONDRINA

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

A - <u>Informações sobre a pesquisa:</u>

Você está sendo convidada a participar, como voluntária, da pesquisa intitulada "Análise da expressão de genes relacionados a células T regulatórias (Tregs) FOXP3+ em pacientes com câncer de mama", que tem por objetivo analisar um determinado tipo de DNA que pode influenciar na imunidade da paciente. Você será esclarecida sobre a pesquisa em qualquer aspecto que desejar. Sua participação não é obrigatória e, a qualquer momento, você poderá desistir de participar e retirar seu consentimento, sem que isso acarrete qualquer penalidade.

B - <u>Procedimento do Estudo</u>

Os procedimentos da pesquisa envolvem a obtenção de 5mL de sangue periférico para análise das células e moléculas do sistema imunológico. O tecido mamário tumoral retirado durante a cirurgia será encaminhado para análise histológica e o restante será utilizado para a realização deste projeto.

C- <u>Confidencialidade da Pesquisa</u>

As informações obtidas através desta pesquisa serão confidenciais e asseguramos o sigilo sobre sua participação. Os dados não serão divulgados de forma a possibilitar sua identificação.
A amostra de sangue obtida, será utilizada para obtenção de DNA o qual após a realização deste projeto, ficará a disposição para utilização de outras pesquisas sob coordenação e gerenciamento do Comitê de Ética em Pesquisa da Universidade Estadual de Londrina, desde que esta Instituição venha futuramente apresentar um Regulamento Institucional para sua utilização. Todos os projetos que utilizem este material posteriormente a este projeto deverão ser submetidos ao Comitê de Ética para reanálise e consentimento da pesquisa a ser realizada.
A participação no estudo não acarretará custos para você e não haverá nenhuma compensação financeira adicional. Você receberá uma cópia deste termo onde consta o telefone e o endereço do coordenador do projeto de pesquisa, podendo tirar suas dúvidas sobre o projeto e sua participação, agora ou a qualquer momento.
A coordenadora do projeto é a Profa. Dra. Maria Angelica Ehara Watanabe, que pode ser encontrada no endereço: Rod. Celso Garcia Cid, 445, Departamento de Ciências Patológicas, Centro de Ciências Biológicas, Universidade Estadual de Londrina, CEP: 86051-970, Tel/Fax: (43) 3371-5729.

D- <u>Consentimento livre esclarecido e informado:</u>

Eu, _____, RG _____, declaro que estou de acordo com as informações contidas neste documento, fui devidamente esclarecido pelo (s) pesquisador (es) dos objetivos e procedimentos da pesquisa de maneira clara e detalhada, e esclareci minhas dúvidas. Concordo em participar voluntariamente desse estudo sendo que poderei retirar meu consentimento a qualquer momento, antes ou durante o mesmo, sem penalidades ou prejuízos no meu atendimento neste serviço.

Londrina, _____ de _____, 20_____.

Assinatura do doador: _____