JULIE MASSAYO MAEDA ODA

POLIMORFISMOS GENÉTICOS E EXPRESSÃO GÊNICA DE *FOXP3* E *TGFB1*:

POSSÍVEIS MARCADORES MOLECULARES NO CÂNCER DE MAMA

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

Orientadora: Prof[a]. Dr[a]. Maria Angelica Ehara Watanabe.
Co-Orientadora: Prof[a]. Dr[a]. Roberta Losi Guembarovski.

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DEDICATÓRIA

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"A tarefa não é tanto ver aquilo que ninguém viu, mas pensar o que ninguém ainda pensou sobre aquilo que todo mundo vê."

(Arthur Schopenhauer)

ODA, Julie Massayo Maeda. **Polimorfismos genéticos e expressão gênica de *FOXP3* e *TGFB1*:** possíveis marcadores moleculares no câncer de mama. 2013. 118 f. Tese de Doutorado – Programa de Pós-Graduação em Patologia Experimental, Departamento de Ciências Patológicas, Centro de Ciências Biológicas (CCB) – Universidade Estadual de Londrina (UEL), 2013.

RESUMO

O câncer de mama é a principal causa de morte por câncer entre mulheres de todo o mundo e sabe-se que o microambiente tumoral contém células do sistema imunológico, células malignas e do estroma circundante. O fator de crescimento de transformação beta (TGFB) é uma citocina que estimula o desenvolvimento de células T regulatórias (Tregs), que são linfócitos T CD4+, e é produzido tanto por estas células quanto por células tumorais. O *FOXP3* codifica uma importante molécula para a função das Tregs, tanto para manutenção da tolerância quanto na regulação da resposta imunológica. Polimorfismos nesses genes (*FOXP3* e *TGFB*) podem levar ao desequilíbrio do sistema imune e mediar o desenvolvimento de doenças graves em humanos. Portanto, o presente estudo teve por objetivo analisar as variantes alélicas A/G rs2232365 e C/A rs3761548 do *FOXP3* e T/C rs1800470 do *TGFB1* entre pacientes e controles, bem como suas expressões gênicas no tecido tumoral em comparação com tecido mamário normal. O estudo de associação caso-controle indicou uma associação positiva para o gene *TGFB1* em relação ao homozigoto CC [OR= 1,88 IC 95% (1,19-2,96)] (p=0,0064) e para os portadores do alelo C [OR= 4,41 IC 95% (1,81-10,76)] (p=0,0005). Nenhuma associação significativa foi encontrada para os genótipos homozigotos dos polimorfismos de *FOXP3,* sendo: GG [OR= 1,07 IC 95% (0,64-1,79)] (p=0,8100) e AA [OR= 0,56 IC 95% (0,18-1,78)] (p=0,3195). Entretanto, quando as variantes alélicas (G + A) deste mesmo gene foram analisadas de forma combinada, foi observada uma associação positiva [OR= 6.18 95% CI (1.67-22.93)] (p=0,0021) em relação à suscetibilidade ao câncer de mama. Em relação às características clínicopatológicas (receptor hormonal, tamanho do tumor, metástase em linfonodos, HER-2 e estadiamento clínico) das pacientes, não foram observadas diferenças significativas em relação a nenhum dos polimorfismos propostos. Entretanto, foi observada uma expressão relativa de 15,06 vezes maior do RNAm de *FOXP3* no tecido tumoral em comparação com um *pool* comercial de RNA de glândula mamária normal. Quando foram comparadas as expressões relativas de *FOXP3* com as variantes alélicas polimórficas, assim como com os parâmetros clínicopatológicos, também não foram observadas diferenças significativas. Uma vez que a superexpressão de *TGFB* no microambiente tumoral pode induzir metástases e que *FOXP3* está envolvido no prognóstico e sobrevida de pacientes com câncer de mama, o nosso estudo sugere que os polimorfismos desses dois genes analisados bem como a expressão de *FOXP3* podem ser considerados marcadores de suscetibilidade na patogênese do câncer de mama.

Palavras Chaves: câncer de mama, polimorfismo genético. *TGFB1*. *FOXP3*. Treg, marcador molecular.

ODA, Julie Massayo Maeda. **Genetic polymorphisms and gene expression of *FOXP3* and *TGFB1*:** possible molecular markers in breast cancer. 2013. 118 p**.** Doctoral Tesis– Post-Graduate Program in Experimental Pathology, Department of Pathological Sciences, Biological Science Center (CCB) – University State of Londrina (UEL), 2013.

ABSTRACT

Breast cancer is the leading cause of cancer death among women worldwide, and it is known that the tumor microenvironment contains immune cells, and the surrounding stroma. The transforming growth factor beta (TGFB) is a cytokine which stimulates the development of regulatory T cells (Tregs) that are CD4+ T lymphocytes and is produced by these cells as well as by tumor cells. The *FOXP3* gene encodes an important molecule for the function of Tregs, both for maintenance of tolerance and in the regulation of immune response. Polymorphisms in these genes (*FOXP3* and *TGFB*) may lead to imbalance of the immune system and mediate the development of important human diseases. Therefore, this study aimed to analyze the allelic variants A/G rs2232365 and C/A rs3761548 of *FOXP3* and T/C rs1800470 of *TGF -B1* between patients and controls, as well as their gene expressions in tumor tissue than in normal breast tissue. The case-control study indicated a positive association between *TGFB1* gene for homozygous CC [OR= 1.88 95% CI (1.19-2.96)] (p=0.0064) and for carriers of the C allele [OR= 4.41 95% CI (1.81-10.76)] (p=0.0005). No significant association was found between *FOXP3* polymorphisms (A/G rs2232365, C/A rs3761548), when analyzed separately GG [OR= 1.07 95% CI (0.64-1.79)] (p=0.8100) and AA [OR= 0.56 95% CI (0.18-1.78)] (p=0.3195), respectively. However, when the allelic variants (G + A) of the same gene were analyzed in combination, a positive association [OR= 6.18 95% CI (1.67-22.93)] (p=0.0021) in relation to susceptibility to breast cancer was observed. In relation the clinical features (hormonal receptor, tumor size, lymph node metastasis, HER-2 and clinical staging) of patients, no significant differences in any of the proposed polymorphisms were observed. However, there was a relative expression of 15.06 fold higher of *FOXP3* in tumor tissue compared to a commercial pool of normal mammary gland RNA. When the relative expression of *FOXP3* was compared with polymorphic allelic variants, as well as with clinicopathological parameters, no significant differences were observed. Since the overexpression of *TGFB* in the tumor microenvironment could induce metastasis and that the *FOXP3* is involved in the prognosis and survival of breast cancer patients, our study suggests that polymorphisms of these two genes and expression of *FOXP3* may be considered biomarkers in the pathogenesis of breast cancer.

Keywords: Breast Cancer. genetic polymorphism. *TGFB1*. *FOXP3*. Treg, molecular marker.

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RESUMEN

El cáncer de mama es la principal causa de muerte por cáncer entre las mujeres en todo el mundo, y se sabe que el microambiente del tumor contiene células inmunitarias, células del cáncer y el estroma circundante. El factor de crecimiento transformante beta (TGFB) es una citocina que estimula el desarrollo de las células T reguladoras (Treg) que son linfocitos T CD4+ y es producido por estas células así como por las células tumorales. El gen *FOXP3* es una molécula importante para la función de las células T reguladoras, tanto em el contexto del mantenimiento de la tolerancia como em la regulación de la respuesta inmune. Los polimorfismos en estos genes (*FOXP3* y *TGFB*) pueden conducir a um desequilíbrio em el sistema inmune y mediar em el desarrollo de enfermedades humanas graves. Por lo tanto, este estúdio tuvo como objetivo analizar las variantes alélicas A/G rs2232365 y C/A rs3761548 del *FOXP3* y T/C rs1800470 del *TGFB1* entre pacientes y controles , así como sus expresiones de genes en el tejido tumoral que en el tejido mamario normal. El estudio de casos y controles demostró una asociación positiva entre el gen de *TGFB*1 homocigotos CC [OR= 1,88 IC 95% (1,19-2,96)] (p=0,0064) y para los portadores del alelo C [OR= 4,41 IC 95% (1,81-10,76)] (p=0,0005). No se encontro asociación significativa entre los polimorfismos *FOXP3* (A/G rs2232365, C/A rs3761548), cuando se analizaron por separado GG [OR= 1,07 IC 95% (0,64-1,79)] (p=0,8100) y AA [OR= 0,56 IC 95% (0,18-1,78)] (p=0,3195), respectivamente. Sin embargo, cuando se analizaron las variantes alélicas (G + A) del mismo gene, la combinación se observo una asociación positiva [OR=6,18 IC 95% (1,67-22,93)](p=0,0021)en relación a la susceptibilidad a cáncer de mama. Al analizar las características clínico (los receptores de la hormona, el tamaño del tumor, la metástasis de los ganglios linfáticos, HER-2 y la estadificación clínica), no se encontró asociación significativa. Sin embargo, cuando se analizaron las variantes alélicas del mismo gen en combinación, se observó una asociación positiva [OR= 6,18 IC 95% (1,67-22,93)] en relación con la susceptibilidad al cáncer de mama. Sin embargo, hubo un aumento de 15,06 veces la expresión relativa de *FOXP3* em el tejido tumoral em comparación con uno *pool* comercial de la glándula mamaria normal. Cuando las expresiones relativa del *FOXP3* se compararon las variantes alélicas polimórficas, así como con los parámetros clínico, también no se observaron diferencias significativas. Sabiendo que el aumento de la expresión de *TGFB* en el microambiente tumoral puede induci rmetástasis y que el *FOXP3* está involucrado em el pronóstico y la supervivencia de pacientes com cáncer de mama, nuestro estúdio sugiere que los polimorfismos de estos dos genes analizados y la expresión de *FOXP3* pueden considerarse biomarcadores em la patogénesis del cáncer de mama.

Palabras-clave: cáncer de mama. polimorfismo genético. *TGFB1*. *FOXP3*. Treg. los marcadores moleculares.

SUMÁRIO

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LISTA DE ABREVIATURAS E SIGLAS

α	Alfa
$AgNO_3$	Nitrato de Prata
AJCC	(*American Joint Committee on Cancer*): Comitê Americano de Câncer
B/B	beta
bp	(*base pair*): pares de base
CD	(*Cluster of Differentiation*): Marcador de Superfície
CDI	Carcinoma Ductal Invasor
CEP	Comitê de Ética em Pesquisa Envolvendo Seres Humanos
CLI	Carcinoma Lobular Invasor
CNS	Conselho Nacional de Saúde
CONEP	Comissão Nacional de Ética em Pesquisa
Ct	*Cycle Threshold*
DEPC	Dietil Pirocarbonato
DNA	(*Desoxyribonucleic Acid*): Ácido Desoxirribonucléico
DNAc	Ácido Desoxirribonucléico Complementar
dNTP	Desoxirribonucleotideo trifosfato
EDTA	(*Ethylene Diamine Tetraacetic Acid*): Ácido Dietilenoaminotetraacetato Dissódico
ER	(*Estrogen Receptor*): Receptor de Estrógeno
FD	Fator de Diluição
FOXP3	*Forkhead box P3*
h	Horas
HCL	Hospital do Câncer de Londrina
IL	Interleucina
INCA	Instituto Nacional de Câncer
kD	Kilodalton
µg	Micrograma
µL	Microlitro

mg	Miligrama
MAP	(*Mitogen Activated Protein*): Proteína Mitogênica Ativadora
MHC	(*Major Histocompatibility Complex*): Complexo de Histocompatibilidade Principal
min	Minutos
mL	Mililitro
mM	Milimolar
NCBI-NIH	*National Center for Biotechnology Information - National Institutes of Health*
NK	*Natural killer*
nm	Nanômetro
ºC	Graus Celsius
PCR	(*Polymerase Chain Reaction*): Reação em Cadeia da Polimerase
PI-3K	Fosfoinositol-3-Quinase
PR	(*Progesterone Receptor*): Receptor de Progesterona
qRT-PCR	*(Real Time quantitative Reverse Transcription PCR*): PCR quantitativo em tempo real
RNA	(*Ribonucleic Acid*): Ácido ribonucléico
RNAm	Ácido Ribonucléico mensageiro
rpm	Rotações por minuto
RT-PCR	*(Reverse Transcription Polymerase Chain Reaction*): Reação em Cadeia da Polimerase via Transcriptase Reversa
18 S	18 ribosomal RNA / S = *Svedberg units*
s	Segundos
SDS	(*Sodium Dodecyl Sulfate*): Dodecil Sulfato de Sódio
SEM	(*Standard Error*): Erro padrão
SNP	*(Single Nucleotide Polymorphism):* Polimorfismo de um Único Nucleotídeo
TCR	(*T Cell Receptor*): Receptor de Célula T

TGFB	(*Transforming Growth Factor Beta*): Fator de Crescimento de Transformação beta
TMEPAI	(*Transforming Growth Factor-Beta Induced Transmembrane Protein*): Proteína Transmembrana Induzida pelo *TGFB*
TNM	Tumor-Nódulo-Metástase
Treg	(*Regulatory T cells*): Células T regulatórias
U	Unidade
UICC	União Internacional de Controle do Câncer
V	Volts
VEGF	(*Vascular Endothelial Growth Factor*): Fator de crescimento do endotélio vascular
XC	Xileno Cianol
%	Porcentagem

INTRODUÇÃO

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 contribuem para a iniciação, crescimento, invasividade e desenvolvimento de metástase (YAQUB; AANDAHL, 2009). A interação entre os tumores e seu microambiente imunológico é complexa sendo fundamental para o desenvolvimento de novos marcadores prognósticos e estratégias terapêuticas (FRIDMAN *et al.*, 2011).

A importância mundial do câncer é inquestionável, uma vez que é a segunda maior causa de mortes no mundo, atrás apenas das doenças cardiovasculares. A incidência de diferentes tipos de câncer tem aumentado tanto nos países desenvolvidos como nos países em desenvolvimento como resultado da crescente exposição a fatores de risco e do aumento da expectativa de vida. Estima-se que, em 2020, o número de casos novos por ano seja da ordem de 15 milhões (INCA/MS, 2007).

Câncer de Mama

O câncer de mama é o tipo de câncer que mais acomete as mulheres em todo o mundo, tanto em países em desenvolvimento quanto em países desenvolvidos (INCA/MS, 2012). O câncer de mama é uma das malignidades mais comuns, representando 22% dos cânceres que afetam as mulheres no mundo. A incidência desta doença é alta no Norte da América, Europa e Austrália comparada a outras regiões, incluindo África do Sul e Leste da Ásia (HAUPT *et al.*, 2010). Em 2014, há previsão, para o Brasil de 56.090 novos casos de câncer da mama, com um risco estimado de 56 casos a cada 100 mil mulheres. Sem considerar os tumores da pele não melanoma, esse tipo de câncer também é o mais frequente nas mulheres das regiões Sudeste (22/100 mil), Sul (21/100 mil), Centro-Oeste (19/100 mil) e Nordeste (20/100 mil). Na região Norte é o segundo tumor mais incidente (17/100 mil) (BRAZIL, 2013).

Os tumores de mama em humanos são tecidos histologicamente complexos, contendo uma variedade de tipos celulares juntamente com células do carcinoma (RONNOV-JESSEN *et al.*, 1996). Dois tipos distintos de células epiteliais são encontrados na glândula mamária humana: células basais (e/ou mioepiteliais) e células epiteliais luminais (TAYLOR-PAPADIMITRIOU *et al.*, 1989; RONNOV-JESSEN *et al.*, 1996). Esses dois tipos celulares

são diferenciados por imunohistoquímica: células epiteliais basais podem ser coradas com anticorpos contra queratina 5 e 6, enquanto células epiteliais luminais coram-se com anticorpos contra queratina 8 e 18 (RONNOV-JESSEN *et al.*, 1996; PEROU *et al.*, 2000).

Classificação Clínica do Câncer de Mama

O Sistema TNM foi desenvolvido por Pierre Denoix em meados de 1942 e representou uma tentativa de classificar o câncer baseando-se nos atributos morfológicos maiores dos tumores malignos que acreditavam influenciar o prognóstico da doença, como: tamanho do tumor primário (T), presença e extensão do envolvimento de nódulos linfáticos regionais (N), e presença de metástases distantes (M). A UICC apresentou a classificação clínica de câncer de mama baseada no Sistema TNM em 1958 e o Comitê Americano de Câncer (AJCC – *American Joint Committee on Cancer*) publicou um sistema de estadiamento de câncer de mama baseado no TNM no seu primeiro manual de estadiamento de câncer em 1977 (BEAHRS *et al.*, 1977). Desde então, revisões regulares têm sido realizadas a fim de promover maiores avanços em diagnósticos e tratamentos. Na revisão de 1987, diferenças entre as versões do AJCC e do UICC no sistema TNM foram eliminadas. Portanto, esta avaliação tem como base a dimensão do tumor (T), a avaliação da extensão aos linfonodos (N) e a presença ou não de metástases à distância (M).

Esta classificação aplica-se apenas aos carcinomas, sendo indispensável a confirmação histológica. Recomenda-se que, quando houver múltiplos tumores, o maior deles seja considerado para definição dos parâmetros e quando houver tumores sincrônicos bilaterais, a classificação de cada um deles será isolada (INCA/MS, 2007).

As classificações conforme o tamanho do tumor (T), comprometimento nodular (N) e metástases (M) estão apresentadas nos quadros em Anexo (Tabelas 5, 6, 7 e 8), além de agrupar as diversas combinações possíveis baseadas no sistema TNM em vigência - 7ª edição (AJCC, 2010).

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 mais às células-tronco, e indicam, 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).

Os tumores de mama são classificados histologicamente de acordo com o sítio de origem da neoplasia, dividindo-se em ductais e lobulares. Os ductais se desenvolvem nos ductos mamários e representam cerca de 80% dos tumores. Os lobulares desenvolvem-se no interior dos lóbulos e representam cerca de 10 a 15% dos casos. Outros subtipos raros representam menos de 10% dos casos diagnosticados por ano (VARGO-GOGOLA; ROSEN, 2007). As pacientes com carcinoma ductal invasivo apresentam maior envolvimento linfático e um pior prognóstico que o verificado nas pacientes com tipos menos frequentes de carcinoma invasivo de mama (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; WITTEKIND, 2002).

O tipo histológico, o grau, o tamanho do tumor, o envolvimento de linfonodos, os receptores hormonais de estrógeno α (ER) e o receptor HER-2 influenciam no prognóstico e na probabilidade de resposta às terapias sistêmicas (D'EREDITA *et al.*, 2001; OLIVOTTO *et al.*, 2005).

Classificação Molecular do Câncer de Mama

A heterogeneidade do câncer de mama em termos de histologia do tumor, apresentação clínica e resposta ao tratamento tem sido analisada a nível molecular pelo perfil de expressão gênica, o qual revela que cada tumor tem uma assinatura molecular única (PEROU *et al.*, 2000; YEHIELY *et al.*, 2006) que pode levar ao descobrimento de novos alvos terapêuticos e tratamentos que possam ser mais efetivos para as pacientes (PEROU *et al.*, 2000). A classificação molecular não substitui, mas sim complementa o diagnóstico anatomopatológico do câncer de mama.

A análise dos perfis gênicos do carcinoma de mama humano pela técnica de microarranjo classifica esta doença em cinco subtipos distintos ou também conhecidos como subtipos moleculares: luminal A, luminal B, mama *normal-like*, superexpressão do receptor

de fator de crescimento epitelial humano (HER-2) e câncer de mama *basal-like* (HAUPT *et al.*, 2010).

O ambiente hormonal conhecidamente influencia o curso da doença (BRISKEN; O'MALLEY, 2010), sendo os cânceres de mama classificados pela expressão de ER e PR (receptor de progesterona), os quais possuem diferentes características clínicas, patológicas e moleculares (ALTHUIS *et al.*, 2004). Os receptores hormonais (estrógeno e progesterona) são expressos tanto no epitélio quanto no estroma mamário (DANIEL *et al.*, 1987), atuando de forma sinérgica a outros fatores de risco, contribuindo assim para a patogênese do câncer de mama por meio de mecanismos relacionados à exposição hormonal (POTTER *et al.*, 1995; ENGER *et al.*, 2000; HUANG *et al.*, 2000; MANJER *et al.*, 2001).

PEROU *et al.* (2000) demonstraram a existência de subtipos moleculares dentro dos cânceres de mama ER positivos e negativos. Os ER positivos são classificados como grupo luminal e os cânceres ER negativos são subclassificados em HER-2, *basal-like* e mama *normal-like*. Entretanto, REIS-FILHO; PUSZTAI (2011) agruparam os subtipos moleculares do câncer de mama baseado em marcadores específicos como os receptores hormonais, a presença ou ausência de expressão de HER-2, Ki67 e citoqueratinas, como representado na Tabela 1.

Tabela 1. Classificação molecular do câncer de mama baseado em marcadores específicos como os receptores hormonais, a presença ou ausência de expressão de HER-2, Ki67 e citoqueratinas.

Classificação do câncer de mama	Marcadores por IHC	Grau Histológico	Proliferação	Outros marcadores	Resultado*	Benefícios da quimioterapia	Classificação PAM50 vs ER/PR/HER-2+
Luminal A	ER+: 91-100% PR+: 70-74% HER-2+: 8-11% Ki67: baixa expressão Marcadores basais: negativo	GI/II: 70–87% GIII: 13–30%	Baixo	FOXA1 high	Bom	Baixo (0–5% pCR)	ER ou PR+/HER2–: 97·4% HER2+: 1·3% ER–/HER2–: 1·3%
Luminal B	ER+: 91-100% PR+: 41-53% HER-2+: 15-24% Ki67: alta expressão Marcadores basais: negativo	GI/II: 38–59% GIII: 41–62%	Alto	FGFR1 e ZIC3 amp	Intermediário/Ruim	Intermediário (10–20% pCR)	ER ou PR+/HER2–: 98·2% HER2+: 1·8% ER– e PR– /HER2–: 0%
Basal-*like*	ER+: 0-19% PR+: 6-13% HER-2+: 9-13% Ki67: alta expressão Marcadores basais: positivo	GI/II: 7–12% GIII: 88–93%	Alto	RB1: low/– CDKN2A: high BRCA1: low/– FGFR2: amp	Ruim	Alto (≥40% pCR)	ER ou PR+/HER2–: 20·0% HER2+: 8·9% ER– e PR– /HER2–: 71·1%
Superexpressão de HER-2	ER+: 29- 59% PR+: 25-30% HER-2+: 66-71% Ki67: alta expressão Marcadores basais: negativo/positivo	GI/II: 11–45% GIII: 55–89%	Alto	GRB7: high	Ruim	Intermediário (25–40% pCR)	ER ou PR+/HER2–: 29·7% HER2+: 45·9% ER– e PR– /HER2–: 24·3%
Normal *breast-like*	ER+: 44-100% PR+: 22-63%	GI/II: 37–80% GIII: 20–63%	Baixo/Intermediário	...	Intermediário	Baixo (0–5% pCR)	ER ou PR+/HER2–:

	HER-2+: 0-13% Ki67: baixa/intermediária expressão Marcadores basais: negativo/positivo						88·9% HER2+: 5·6% ER– e PR– /HER2–: 5·6%
Claudina baixa	ER+: 12-33% PR+: 22-23% HER-2+: 6-22% Ki67: intermediária expressão Marcadores basais: positivo/negativo	GI/II: 62–23% GIII: 38–77%	Intermediário/Alto	CDH1: low/– Claudins: low/–§	Intermediário	Intermediário (25–40% pCR)	Subtipo não incluso no PAM50
Molecular apócrina	ER: negativo PR: negativo HER-2: positivo/negativo Ki67: alta expressão Marcadores basais: negativo/positivo	Predominantemente GII/GIII	Alto	Receptor Andrógeno: +	Ruim	Não examinado	Subtipo não incluso no PAM50

ER: Receptor estrógeno
PR: Receptor progesterona
IHC: Imunohistoquímica
G: Grau Histológico
pCR: Resposta patológica completa após quimioterapia neoadjuvante
–/+: Predominantemente negativo
+/–: Predominantemente positivo
* Regime de terapia convencional

*Fonte:*REIS-FILHO; PUSZTAI (2011) modificado.

O prognóstico e a sensibilidade às quimioterapias são diferentes para os distintos subgrupos moleculares. Os cânceres luminais tendem a ter sobrevida mais favorável em longo prazo comparado aos outros subtipos, enquanto os tumores positivos para o HER-2 e os *basal-like* são mais sensíveis à quimioterapia (SORLIE *et al.*, 2001; ROUZIER *et al.*, 2005).

Sistema imune e câncer

Sabe-se que a patogênese do câncer é iniciada e modulada pela interação tumor-hospedeiro e que, componentes do sistema imune atuam tanto na defesa como contribuem para a progressão tumoral (YAQUB; AANDAHL, 2009). O entendimento dessa cooperação é de fundamental importância para o desenvolvimento de novos marcadores prognósticos e estratégias terapêuticas (FRIDMAN *et al.*, 2011).

Todo carcinoma humano induz uma resposta imune em seu microambiente. Geralmente, esta reação é considerada não efetiva para destruir as células do câncer, contudo, nos últimos anos, algumas evidências têm demonstrado a importância da infiltração de células do sistema imunológico, tais como linfócitos e macrófagos (OSTRAND-ROSENBERG; SINHA, 2009; DE LA CRUZ-MERINO *et al.*, 2013).

Os mecanismos que inter-relacionam os processos inflamatórios, imunidade e câncer têm sido muito discutidos. Importantes componentes nesta integração são as citocinas produzidas pelas células ativadas do sistema imune inato ou adaptativo que estimulam o crescimento tumoral e a progressão do câncer. Além disso, mediadores solúveis produzidos pelas células tumorais recrutam e ativam células inflamatórias que também estimulam a progressão. Entretanto, as células inflamatórias também podem produzir citocinas, como o IFN-ɣ, que limitam o crescimento do tumor (LIN; KARIN, 2007).

As imunidades inata e adaptativa desempenham importantes papéis na imunovigilância e destruição tumoral (WANG, 2006). Contudo, os tumores têm a capacidade de evadir do reconhecimento imunológico, induzir disfunção das células imunes e escapar da vigilância imunológica por numerosos mecanismos (WHITESIDE, 2008), tais como diminuição de moléculas de MHC (Complexo de Histocompatibilidade Principal) tipo I e de moléculas co-estimulatórias como B7 e secreção de citocinas imunossupressoras (IL-10 e TGF-B1).

Estudos têm relatado que o infiltrado tumoral de células imunes, em particular as células T CD8+, foi correlacionado com a sobrevivência dos pacientes (SATO *et al.*, 2005),

enquanto a presença de outras células, tais como as células T regulatórias (Treg) estão correlacionadas com um pior prognóstico (CURIEL *et al.*, 2004).

Forkhead Box P3 (FOXP3)

As células Tregs constituem uma linhagem de células T CD4+ que desempenham um papel indispensável na tolerância imunológica aos autoantígenos e na supressão de respostas imunes excessivas, consideradas deletérias ao hospedeiro. No entanto, estas células também limitam as respostas benéficas por suprimirem a imunidade antitumoral (SAKAGUCHI *et al.*, 2008; VIGNALI *et al.*, 2008; TOKER; HUEHN, 2011).

A maioria das células T CD4+ reguladoras é normalmente caracterizada por uma elevada expressão do receptor de superfície da interleucina 2 (IL -2) cadeia α (CD25) e seu fenótipo é geralmente aceito como $CD4^{+}CD25^{hi}CTLA4^{+}GITR^{+}FOXP3^{+}CD45RO^{+}CD45RA^{-}CD69^{-}Ki\text{-}67^{-}$ (SAKAGUCHI, 2005; BETTS *et al.*, 2006). O FOXP3 é expresso primariamente nessa subpopulação de células T CD4+ que expressam CD25, conhecido como células Treg (COFFER; BURGERING, 2004; CAMPBELL; ZIEGLER, 2007; EASTELL *et al.*, 2007). FOX (*forkheadbox*) agora é usado como o símbolo para todos os fatores de transcrição *forkhead* dos cordados. A análise filogenética resultou na definição de 15 classes para todas as proteínas FOX conhecidas, assim estes fatores de transcrição são classificados em termos de estrutura e não de função. O FOXP3 (fator de transcrição *forkhead box P3*) é um membro da família de fator de transcrição *forkhead winged*-hélice e possui três domínios funcionais: um domínio *zinc-finger* C2H2 (aminoácidos 200-223), um domínio zíper-leucina (aminoácidos 240-261) e um domínio carboxi-terminal *forkhead* (aminoácidos 338-421), como observado na Figura 1. As análises das respectivas sequências revelaram que essas três regiões não-codificadoras altamente conservadas estão sujeitas a modificações epigenéticas e estão envolvidas na regulação da transcrição do *FOXP3* (HUEHN *et al.*, 2009).

O *FOXP3* foi identificado em 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). O tamanho completo do *FOXP3* humano é de 1296 pb e foi relatado que consiste em 11 exons. Este gene situa-se no braço curto do cromossomo X (Xp11.23), o qual está sujeito à inativação cromossômica, e codifica uma proteína de 431 aminoácidos (GAMBINERI *et al.*, 2003; FONTENOT *et al.*, 2005; BAN *et al.*, 2007; TORGERSON; OCHS, 2007).

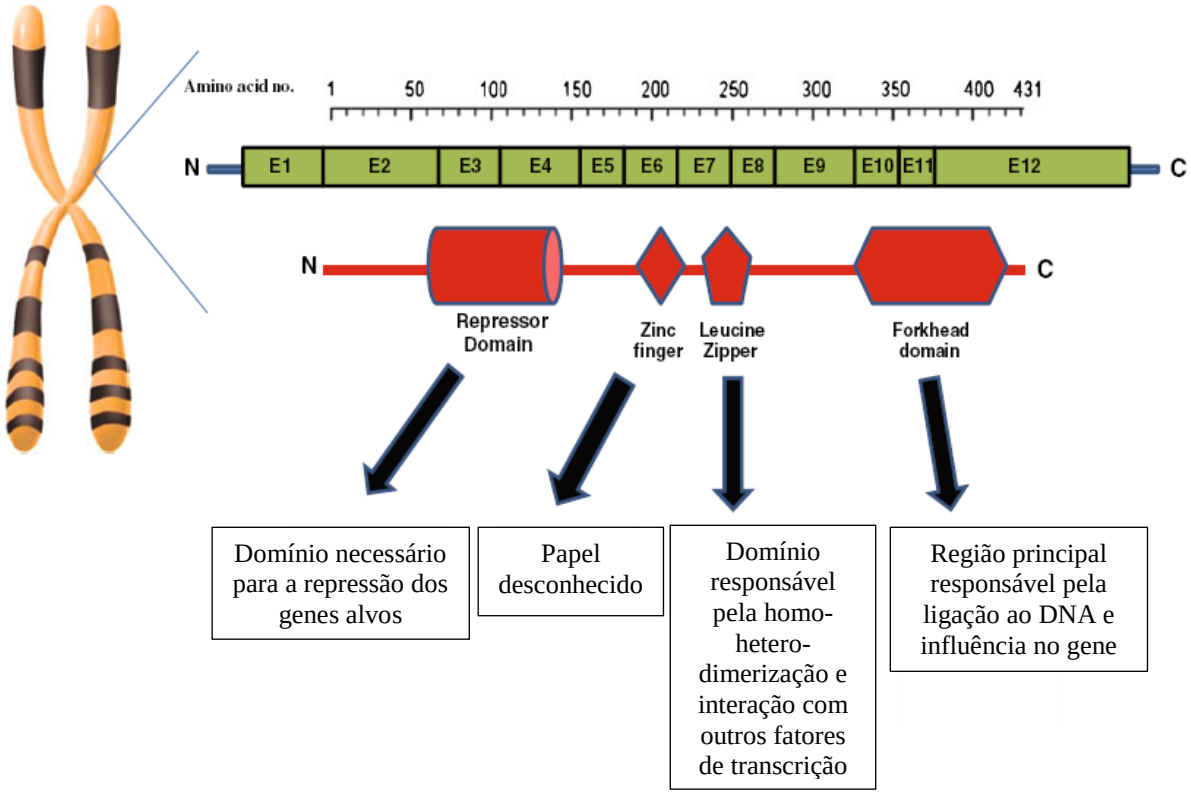

Figura 1 Representação esquemática da estrutura e das funções do gene *FOXP3* humano. O gene FOXP3 está localizado no cromossomo X na posição Xp11.23. O gene completo do *FOXP3* consiste de 12 éxons, dos quais 11 são codificados à proteína. Quatro domínios principais compõem o *FOXP3*, os quais cada domínio contribui diferentemente para sua função global. Fonte: DOUGLASS *et al.* (2012) modificado.

Polimorfismos têm sido descritos em várias regiões do gene *FOXP3*, tais como a região promotora(Tabela 2), intrônica (Tabela 3) e exônica (Tabela 4) associadas às doenças humanas (ODA *et al.*, 2013), como pode ser observado abaixo:

Tabela 2 Polimorfismos do FOXP3 na região promotora

Polimorfismo	Doença	Ano	País
rs2232365	Doença de Crohn	2005	USA
	Doença de Graves e Addison	2006	UK
	Psoríase	2010	China
	Aborto espontâneo recorrente sem explicação	2012	China
rs3060515	Rinite alérgica	2009	China
	Lupus Eritematoso Sistêmico	2011	Taiwan
rs3761547	Artrite Juvenil Idiopática	2007	UK
	Rinite alérgica	2009	China
	Doença de Hashimoto e Graves	2010	Japão
rs3761548	Doença de Crohn	2005	USA
	Câncer de Mama	2009	Israel
	Rinite alérgica	2009	China
	Atopia	2010	Holanda
	Psoríase	2010	China
	Doença de Hashimoto e Graves	2010	Japão
	Endometriose e Infertilidade	2011	Brasil
	Rinite alérgica	2011	Hungria
	Lupus Eritematoso Sistêmico	2011	Taiwan
	Aborto espontâneo recorrente sem explicação	2012	China
rs3761549	Doença de Graves e Addison	2006	UK
	Doença de Hashimoto e Graves	2010	Japão
	Endometriose e Infertilidade	2011	Brasil
rs5902434	Psoríase	2010	China
	Aborto espontâneo recorrente sem explicação	2012	China
(GT)n	Diabetes Tipo I	2003	Japão
	Diabetes Tipo I	2004	Itália
	Lupus Eritematoso Sistêmico, Artrite reumatóide	2005	Espanha
	Colite Ulcerativa, Doenças de Crohn e Celíaca, Diabetes Tipo I	2006	Noruega
	Doença de Graves e Addison	2006	UK
	Tireóide autoimune	2007	Japão
	Diabetes Tipo I	2007	Japão

Tabela 3 Polimorfismos do FOXP3 em região intrônica

Polimorfismo	Doença	Ano	País
rs2232366	Endometriose e Infertilidade	2011	Brasil
rs2232367	Artrite Juvenil Idiopática	2007	UK
rs2232368	Endometriose e Infertilidade	2011	Brasil
rs2280883	Doença de Crohn	2005	USA
	Doença de Graves e Addison	2006	UK
	Artrite Juvenil Idiopática	2007	UK
	Psoríase	2010	China
	Endometriose e Infertilidade	2011	Brasil
rs2294019	Atopia	2010	Holanda
rs2294020	Artrite Juvenil Idiopática	2007	UK
	Câncer de mama	2009	Israel
rs2294021	Doença de Graves e Addison	2006	UK
	Atopia	2010	Holanda
	Aborto espontâneo recorrente sem explicação	2012	China
rs4824747	Artrite Juvenil Idiopática	2007	UK
	Diabetes Tipo I	2010	UK
rs5906761	Câncer de mama	2009	Israel
	Atopia	2010	Holanda
rs6609857	Doença de Graves e Addison	2006	UK
	Artrite Juvenil Idiopática	2007	UK
	Atopia	2010	Holanda
	Síndrome IPEX	2001	USA
(TC)n	Diabetes Tipo I	2003	Japão
	Doença de Graves e Addison	2006	UK
	Diabetes Tipo I	2004	Itália
	Diabetes Tipo I e Doença Celíaca	2006	Noruega
	Tireóide autoimune	2007	Japão

Tabela 4 Polimorfismos do FOXP3 em região exônica

Éxon	Doença	Ano	País
1	Síndrome IPEX	2006	Itália/França/USA
12	Doença autoimmune e risco de desenvolvimento de inibidores de FVIII	2010	Itália
2–12	Leucemias agudas	2011	Coréia

Segundo HOOGENDOORN *et al.* (2003), os promotores estão envolvidos na iniciação da transcrição e são, portanto, os elementos de atuação *cis* mais importantes que

regulam a expressão dos genes e podem conter os polimorfismos funcionalmente mais relevantes.

Transforming Growth Factor beta 1 (TGFB1)

Uma variedade de citocinas, quimiocinas e fatores de crescimento é produzida no ambiente tumoral por diferentes tipos celulares através de interações complexas e regulação de diferenciação, ativação, função e sobrevivência dos inúmeros tipos celulares. A interação destas moléculas com seus respectivos receptores forma uma rede global no local do tumor, que é responsável pela progressão e propagação das células tumorais ou indução de uma resposta imune antitumoral e rejeição do tumor (SHURIN *et al.*, 2006).

Uma citocina dimérica que parece ter envolvimento nos cânceres é o fator de crescimento de transformação beta (TGFB), que é expressa por diferentes tecidos (BLOBE *et al.*, 2000), tais como córtex da adrenal, megacariócitos e outras células da medula óssea, miócitos cardíacos, condrócitos, túbulos renais distal, ovário, células coriônicas da placenta, cartilagem, coração, pâncreas, pele e útero (THOMPSON *et al.*, 1989). Tem sido relatado que o TGFB está superexpresso em muitos tumores, como o câncer de colo retal (KEMIK *et al.*, 2013), gliomas (ZAGZAG *et al.*, 2005), próstata (IVANOVIC *et al.*, 2006), pulmão (KANG *et al.*, 2006), rim (HELMY *et al.*, 2007) e os de mama (SUN *et al.*, 2013), e parece estar relacionado à transformação e progressão tumorais (FRIESS *et al.*, 1993; COMERCI *et al.*, 1996; EDER *et al.*, 1996; TSUSHIMA *et al.*, 1996).

O TGFB é um membro de uma ampla família de polipeptídeos multifuncionais secretados que são potentes reguladores do crescimento celular, diferenciação e produção de matriz extracelular (MASSAGUE, 1998; DERYNCK *et al.*, 2001). É uma das proteínas mais pleiotrópicas e multifuncionais do sistema imune. Afeta processos que variam desde a regulação da diferenciação e crescimento celular à inflamação, cicatrização, formação óssea e ainda contribui para a patogênese de diversas doenças, como as autoimunes (COHEN, 2003; LUDVIKSSON; GUNNLAUGSDOTTIR, 2003) e o câncer (TANG *et al.*, 2003; TSUJI *et al.*, 2003).

Esta citocina foi inicialmente identificada e nomeada baseada em sua habilidade de estimular o crescimento de fibroblastos em ágar, mas atualmente é mais estudada como proteína inibitória do crescimento (LEE; BAE, 2002).

A função do TGFB no sistema imune é a manutenção da tolerância via regulação da proliferação, diferenciação e sobrevivência dos linfócitos. Além disso, controla a iniciação e a

resolução da resposta inflamatória pela regulação da quimiotaxia, ativação e sobrevivência de linfócitos, células *natural killer* (NK), células dendríticas, macrófagos, mastócitos e granulócitos. A atividade regulatória é modulada pelo estado de diferenciação e pela presença de citocinas inflamatórias e moléculas co-estimulatórias (LI *et al.*, 2006).

O TGFB é um biomarcador associado à progressão do câncer de mama e é conhecido por atuar tanto como supressor quanto por estimular a progressão do tumor (DERYNCK *et al.*, 2001; SAHA *et al.*, 2004). Os efeitos autócrino e parácrino do TGFB nas células e no microambiente tumoral têm influências positiva e negativa no desenvolvimento do câncer. Em células normais, o TGFB, age através da ligação com os receptores presentes na superfície e leva à ativação de vias de sinalização que podem ser dependentes ou não de Smads. Os principais efeitos desta interação são: parada do ciclo celular na fase G1 para inibir a proliferação, indução da diferenciação ou promoção da apoptose. Durante a transformação de uma célula, vários componentes da sinalização da via do TGFB encontram-se mutados, tornando a célula resistente aos efeitos desta citocina. As células resistentes à ação do TGFB proliferam de forma não regulada, assim como as células do estroma (fibroblastos) que rodeiam o tumor primário em formação. Em seguida, há um aumento na produção de TGFB, que atua tanto na matrix extracelular, no estroma circundante, nas células do sistema imunológico, nas células endoteliais e no músculo liso, levando à imunossupressão e angiogênese, o que favorece a capacidade de invasão do tumor (BLOBE *et al.*, 2000).

A família de proteínas do TGFB é altamente conservada evolutivamente (ZHANG *et al.*, 2006). Existem três isoformas conhecidas do TGFB (TGFB1, TGFB2 e TGFB3) expressas nos tecidos de mamíferos. Todas funcionam pelas mesmas vias de sinalização (CHEIFETZ *et al.*, 1987; MITTL *et al.*, 1996).

Os TGFBs são sintetizados como precursores inativos que contem a região pré (peptídeo sinal) e a região pró (LAP - *Latency Associated Peptide* - complexado à porção N-terminal do peptídeo). O processamento da forma inativa começa com a clivagem proteolítica que remove o peptídeo sinal da forma pré-pro-*TGFB*. Após a dimerização, o *TGFB* é clivado por proteases como a furina na porção C-terminal do peptídeo maduro e na porção N-terminal do LAP. *TGFB* com LAP formam o SLP (*small latent complexes*) que são transportados para a matrix extracelular onde podem sofrer ligação covalente com proteínas ligadoras de *TGFB* latente (LTBP – *latente TGFB binding protein*) para formar o LLC (*large latente complex*). O LTBP é capaz de conectar a forma inativa do *TGFB* às proteínas da matrix extracelular. Esta interação é adicionalmente suportada por ligações covalentes. A ativação do *TGFB* começa com a liberação do LCC da matrix extracelular pelas proteases. Assim, a proteína madura é

clivada do LTBP pela trombospondina (TSP) *in vivo* e pelas condições ácidas ou pela plasmina *in vitro*. Uma vez que um membro da família do *TGFB* é liberado da matriz extracelular, ele se torna capaz de sinalizar (KUBICZKOVA *et al.*, 2012).

Na maioria das células três tipos de proteínas de superfície celular medeiam a sinalização do *TGFB*: o receptor *TGFB* I (TBRI), II (TBRII) e III (TBRIII) (CHEIFETZ *et al.*, 1986; CHEIFETZ *et al.*, 1987).

O *TGFB*2 foi primeiramente descrito em células de glioblastoma humano. Sabe-se que esta citocina é capaz de suprimir o crescimento de linfócitos T dependentes de IL-2. Desse modo, foi nomeado fator supressor de célula T derivado do glioblastoma (G-TsF). Fisiologicamente, o *TGFB*2 é expresso por neurônios e células astrogliais no sistema nervoso embrionário (FLANDERS *et al.*, 1991). A conformação estrutural madura das formas *TGFB1* e *TGFB*2, consiste de 112 aminoácidos na porção C-terminal, e, eles apresentam cerca de 71% de similaridade (TEN DIJKE *et al.*, 1988).

A isoforma TGFB3, foi isolada de um banco de DNA complementar (DNAc) de linhagem celular de rabdomiosarcoma humano, e, demonstra cerca de 80% de similaridade com TGFB1 e TGFB2 (KAARTINEN *et al.*, 1995; PROETZEL *et al.*, 1995).

O TGFB1 é a citocina mais abundante (ZHENG, 2009) e seu gene está localizado no cromossomo 19q13 (FUJII *et al.*, 1986). Muitos polimorfismos neste gene têm sido relatados (CAMBIEN *et al.*, 1996), mas destes, dois são comumente estudados, o T+869C (rs1800470; rs1982073; T29C; Leu10Pro), que está no exon1 e pode levar a uma substituição de uma leucina para uma prolina no códon 10 e o C-509T (rs1800469), que está em uma região promotora (YUAN *et al.*, 2009; ZHENG, 2009). Ambos os polimorfismos estão associados com um aumento no nível plasmático de TGFB1 (GRAINGER *et al.*, 1999; DUNNING *et al.*, 2003). KIRSHNER *et al.* (2006) verificaram que indivíduos com o alelo prolina (alelo C) possuem aumento de 2,8 vezes na secreção de TGFB1 comparada ao alelo leucina *in vitro*. O polimorfismo T+869C está associado à redução da sobrevida livre da doença (SHU *et al.*, 2004; GONZALEZ-ZULOETA LADD *et al.*, 2007).

A superexpressão de TGFB1 foi demonstrada em vários tumores, incluindo os de mama, podendo contribuir para a transformação e progressão tumoral (FRIESS *et al.*, 1993; COMERCI *et al.*, 1996; EDER *et al.*, 1996; TSUSHIMA *et al.*, 1996). Os mecanismos relacionados à transformação e progressão são os seguintes: (1) a produção de TGFB1 pelas células tumorais pode estimular o crescimento tumoral através da promoção da angiogênese e evasão da vigilância imune (ROBERTS *et al.*, 1988; UEKI *et al.*, 1992); (2) TGFB1 pode promover o acúmulo de glicoproteínas da matriz extracelular e adesão proteica das células, e

consequentemente facilitar o potencial metastático do tumor (MASSAGUE *et al.*, 1992); (3) estudos *in vitro* têm demonstrado que a indução da secreção de TGFB1 pode aumentar a mobilidade celular e a produção de protease (SAMUEL *et al.*, 1992); (4) superexpressão de uma proteína transmembrana induzida pelo TGFB em muitos cânceres, a *Transforming Growth Factor-Beta (TGFBeta) Induced Transmembrane Protein* (TMEPAI), a qual converte o TGFB de supressor tumoral para promotor; e (5) recentemente, foi descoberto que o TGFB induz a expressão de *Forkhead Box P3*(*FOXP3)* em células T CD4+CD25+*FOXP3- naïve* e as converte em células T regulatórias (Tregs) *FOXP3*+ (CHEN *et al.*, 2003; ZHANG *et al.*, 2006).

A sinalização intracelular do *TGFB* é complexa e diferentes vias podem ser ativadas. O *TGFB* inicia suas diversas respostas celulares por ligação e ativação de receptores de superfície celular específico que têm atividade intrínseca serina/treonina quinase. Estes incluem as vias dependentes de Smad e independente de Smad, tais como as vias da *Mitogen Activated Protein* (MAP) quinase e fosfoinositol-3-quinase (PI-3K). A via Smad parece ser o principal sinal de transdução do *TGFB* (BUCK; KNABBE, 2006) e corresponde à via inibitória para o crescimento tumoral (efeitos supressores). Os efeitos intracelulares de sinalização de TGFB são ativados por receptores e translocação para o núcleo, onde regulam a transcrição. A fosforilação do receptor ativado por Smads (R-Smads) leva à formação de complexos com o mediador comum Smad (Co-Smad) que são importadas para o núcleo através da regulação dos genes de TGFB. Embora este caminho seja inerentemente simples, interações combinatórias no receptor heteromérico e complexos Smad, a interação com o receptor e a interação das proteínas Smad, assim como a cooperação com fatores de transcrição sequência específica permitem versatilidade e diversificação de resposta pelos membros da família do *TGFB* (MOUSTAKAS *et al.*, 2001; DERYNCK; ZHANG, 2003).

A ativação de vias independentes de Smad, juntamente com a perda das funções supressoras de tumores do TGFB, são importantes para suas funções pro-oncogênicas (NAGARAJ; DATTA, 2010). Em muitos cânceres de mama humanos, a perda do receptor de TGFB ou a expressão do gene associada ao Smad leva ao aumento dos níveis dessa citocina no microambiente tumoral e é suficiente para anular os efeitos supressores tumorais e induzir mobilidade mesenquimal e fenótipo invasivo (TAN *et al.*, 2009). Estes fenômenos ocorrem principalmente devido a causas como a imunossupressão, a degradação da matriz extracelular, transição do epitélio-mesenquimal e angiogênese, que promovem invasão de células tumorais e metástase (JONES *et al.*, 2009).

Um grande esforço tem sido realizado para a identificação de mutações genéticas que desempenham um papel importante na predisposição genética para a progressão de doenças incluindo os cânceres, tais como mutações no gene *BRCA1* e *BRCA2* (genes considerados de alta penetrância) no câncer de mama (MIKI *et al.*, 1994; WOOSTER *et al.*, 1995). No entanto, é provável que um vasto número de genes considerados de baixa penetrância (FGFR2, TOX3, LSP1, MAP3K1, TGFB1, 2q35 e 8q) contribua para a suscetibilidade ao câncer, sendo, portanto, responsáveis também pelo desenvolvimento da doença (WOOSTER *et al.*, 1995; BLACKWOOD; WEBER, 1998; NATHANSON; WEBER, 2001; PHAROAH *et al.*, 2002; RIPPERGER *et al.*, 2009). Existe, desta forma um interesse considerável na identificação de polimorfismos frequentes na população, de baixa ou moderada penetrância. Assim, nos cânceres em que as respostas imunes antitumorais ocorrem, polimorfismos genéticos em produtos que regulam a resposta imunológica são grandes candidatos a investigação (BATEMAN; HOWELL, 1999).

OBJETIVOS

Objetivo Geral

Analisar as variantes alélicas A/G rs2232365 e C/A rs3761548 do FOXP3 e T/C rs1800470 do *TGFB1* em pacientes com câncer de mama e controles livres de neoplasia, bem como determinar as expressões desses mesmos genes por PCR quantitativo em tecido mamário, tumoral e normal.

Objetivos Específicos

- ✓ Obter amostras de sangue periférico de pacientes com câncer de mama e controles livres de neoplasia, assim como obter os dados clinicopatológicos das pacientes, determinar as frequências genotípicas dos polimorfismos A/G rs2232365 e C/A rs3761548 do gene *FOXP3* e T/C rs1800470 do *TGFB1* nos grupos analisados, realizar um estudo de associação caso-controle envolvendo as variantes alélicas dos genes *FOXP3* e *TGFB,* tanto de forma isolada quanto combinada e estimar uma associação entre a presença das variantes alélicas em ambos os genes com os parâmetros clinicopatológicos das pacientes.

- ✓ Obter amostras de tecido mamário tumoral e normal, determinar as frequências genotípicas dos polimorfismos A/G rs2232365 e C/A rs3761548 do gene *FOXP3* e T/C rs1800470 do *TGFB,* assim como determinar a expressão gênica de *TGFB1* e *FOXP3* nos espécimes adquiridos por PCR em tempo real e comparar a expressão gênica e os polimorfismos genéticos de *TGFB1* e *FOXP3* entre si e com os dados clínicopatológicos das pacientes.

PRODUÇÃO BIBLIOGRÁFICA

ARTIGO 1

Transforming Growth Factor B (*TGFB*) and Regulatory T Cells (Treg): The Interface of Tumor and Host Immunity

EUROPEAN JOURNAL OF CLINICAL & MEDICAL ONCOLOGY REVIEW ARTICLE

Transforming Growth Factor β (TGF-β) and Regulatory T Cells (Treg): The Interface of Tumor and Host Immunity

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ABSTRACT

Signals from the microenviroment have a profound influence on the maintenance or progression of cancers. It is known that the transforming growth factor beta (TGF-β) can act as both a tumor suppressor and as a significant stimulator of tumor progression, invasion, and metastasis. TGF-β is an immunosuppressive cytokine produced by tumor cells and immune cells that can polarize many components of the immune system. In addition, TGF-β has been strongly implicated in the control of mammary epithelial growth and in breast cancer cells. Although T cells present the most important immunological response in tumor growth in the early stages of cancer, they become suppressive $CD4^+$ and $CD8^+$ T regulatory cells (Tregs) in the presence of TGF-β stimulation and interactions with tumor cells, thus promoting rather than inhibiting cancer development and progression. Therefore, the cytokine profile from tumor microenvironment, mainly TGF-β and Tregs in a coordinated manner, provides a mechanism that has crucial function in tumorigenesis process.

Keywords: TGF-β, T regulatory cells, tumor microenvironment, breast cancer

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IMMUNOSURVEILLANCE OF TUMORS

Tumors have the capacity to avoid immune recognition, induce immune cell dysfunction, and escape from immune surveillance by numerous mechanisms.[1] Innate and adaptive immunity play important roles in immunosurveillance and tumor destruction.[2] Innate immune cells, including NK, macrophages, NKT, and γd T cells, were shown to play a critical role in protecting a host against cancer.[3] Adaptive immunity is involved in the elimination of pathogens, transforms tumor cells in the late phase of host defense, and generates more specific immunity and immunological memory. Several recent studies have reported that tumor-infiltrating immune cells, in particular $CD8^+$ T cells, were correlated with patient survival,[4] whereas the presence of other immune cells such as Treg cells is correlated with poor prognosis.[5]

Transforming growth factor β (TGF-β) plays an important role in various cell functions, including cell proliferation, differentiation, extracellular matrix remodeling, and embryonic development.[6,7] There are three mammalian isoforms of TGF-β (TGF-β1, TGF-β2, and TGF-β3), and each is encoded by a single gene and expressed in both a tissue-specific and developmentally regulated fashion. The TGF-β1 locus was assigned to 19q13.1,[8] the TGF-β2 gene maps to 1q41, and the TGF-β3 gene is located at 14q24 in man.[9] All three isoforms bind to the same cell surface receptors,[10] and they have been studied intensely with regard to their role in the regulation of tumor progression.[11–13]

In recent years, it has become evident that Regulatory T cells (Tregs) represent an integral part of the immune system and play an important role in both self and acquired immunological tolerance. Tregs consist predominantly of $CD4^+$ cells and can be divided into two principal subsets: naturally occurring, thymus-derived natural $CD4^+$ cells that express CD25, the α chain of the interleukin 2 (IL-2) receptor (nTregs)[14,15] and adaptive $CD4^+CD25^+$ cells that are induced from $CD25^-$ precursors in peripheral lymphoid organs (iTregs).[16] nTregs primarily develop in response to self-antigens expressed in the thymus and iTregs by environmental antigens presented by dendritic cells (DCs) in peripheral lymphoid organs.[17,18] Thus, they may control immune tolerance to innocuous environmental antigens such as those derived from commensal flora.[19]

Today it is known that these molecules act in a coordinated manner, because TGF-β stimulates the development of Tregs and also is a kind of cytokine produced by them; consequently, this interaction has important implications in tumor progression.

TGF-β Signaling

TGF-β isoforms are translated as prepropeptide precursors with an N-terminal signal followed by the prodomain and mature domain and are secreted as biologically latent forms.[20] Latent TGF-β consists of the 25-kDa mature protein in a noncovalent association with the propeptide to which one of several latent TGF-β-binding proteins is linked, called latency-associated peptide (LAP) that contains the signal

sequence for secretion.[10] The TGF-β ligands are secreted and often found in the extracellular matrix as inactive complexes.[21] This complex can be activated through many mechanisms, including ionizing radiation, reactive oxygen free radicals,[22] proteolytic cleavage, interaction with integrins, pH changes in the local environment,[23] or by a number of mechanisms including the expression of many proteins.[24,25]

Intracellular TGF-β signaling is complex and many different pathways can be activated. TGF-β initiates its diverse cellular responses by binding to and activating specific cell surface receptors that have intrinsic serine/threonine kinase activity. These include the Smad-dependent and Smad-independent pathways such as the MAP kinase pathways and phosphoinositol-3-kinase.

The Smad pathway appears to be the major TGF-β signal transduction[26] and it corresponds to the inhibitory pathway to tumor growth (suppressive effects). The intracellular effectors of TGF-β signaling are activated by receptors and translocate into the nucleus, where they regulate transcription. Phosphorylation of receptor-activated Smads (R-Smads) leads to the formation of complexes with the common mediator Smad (Co-Smad) that are imported to the nucleus gene regulation by TGF-β. Although this pathway is inherently simple, combinatorial interactions in the heteromeric receptor and Smad complexes, receptor-interacting and Smad-interacting proteins, and cooperation with sequence-specific transcription factors allow substantial versatility and diversification of TGF-β family responses.[27,28]

It is known that CLIC4 is a highly conserved member of the chloride intracellular channel family of proteins and participates in a wide variety of signaling activities. This protein is largely cytoplasmic but translocates to the nucleus upon a variety of stimuli, including TGF-β. Nuclear resident CLIC4 causes growth arrest, terminal differentiation, and apoptosis. Recently, it was discovered that TGF-β causes CLIC4 to associate with Schnurri-2 (a transcription factor with a role in TGF-β pathway and a function as a cotransporter of CLIC4 to the nucleus in response to TGF-β). The nuclear function of CLIC4 is enhancing TGF-β signaling by associating with phospho-Smad2 and 3 and preventing their dephosphorylation. The revelation of the involvement in TGF-β signaling presents Schnurri-2 and CLIC4 as a unique combination of novel players in a critical pathway, controlling diverse aspects of cell behavior and suggest that these two proteins may serve as targets for modifying TGF-β signaling.[29]

The activation of Smad-independent pathways, coupled with the loss of tumor-suppressor functions of TGF-β, is important for pro-oncogenic functions.[30] In most human breast cancers, lowering of the TGF-β receptor or Smad gene expression combined with increased levels of TGF-β in the tumor microenvironment is sufficient to abrogate TGF-β tumor suppressive effects and to induce a mesenchymal, motile, and invasive phenotype.[31] These phenomena occur especially from causes such as immunosuppression, extracellular matrix degradation, epithelia to mesenchymal transition, and angiogenesis that promotes tumor cell invasion and metastasis.[32]

Thus, the activation of Smad-dependent and independent pathways, together with the interactions derived from the presence or absence of other parallel signaling cascades, determines the functional response to TGF-β stimulation *in vitro* or *in vivo*.[33]

TGF-β and Regulatory T cells

The precise mechanisms by which Tregs suppress immune cell functions remain unclear, and there are reports of both direct inhibition through cell–cell contact and indirect inhibition through the secretion of anti-inflammatory mediators such as TGF-β.

Both nTregs and iTregs share a similar phenotype and have a contact-dependent mechanism of action that is poorly understood. nTregs express cytotoxic T lymphocyte antigen 4 (CTLA-4), gluccorticoid-induced tumor necrosis factor receptor (GITR)), CCR4 (chemokine receptor), and CD62L, and most are previously activated cells. iTregs induced with IL-2 and TGF-β have a similar phenotype except that they are generated from naive $CD4^+$ cells and require additional stimulation to develop memory markers.[34]

Several groups reported that TGF-β induces $CD4^+$ cells to express the Foxp3 in the generation of nTregs,[35,36] and there is also evidence that the presence of TGF-β induces the differentiation of iTreg cells.[35,37,38] TGF-β-induced iTreg cell differentiation is in part mediated by the recruitment of its downstream transcription factor Smad3 to a Foxp3 enhancer element and the consequent induction of Foxp3 gene expression.[39]

Thus, TGF-β has been identified as the most important cytokine supporting Foxp3 expression and Treg development[40,37,41] but other studies have suggested a positive or a negative regulatory role of other cytokines in Treg generation.[42,34,43] It is clear that two inhibitory cytokines, IL-10 and IL-35, are key mediators of Treg-cell function. Although they are all inhibitory, the extent to which they are used in distinct pathogenic and/or homeostatic settings differs, suggesting a nonoverlapping function, but this hypothesis needs further refinement.[44]

Tregs expressing Forkhead box P3 (FoxP3) are key mediators of peripheral tolerance and suppress undesirable immune responses. It was verified that Tregs bear with higher reactivity than other T cells to the selecting ligand in the thymus even after negative selection by the ligand. This broad repertoire and high self-reactivity of $CD25^+CD4^+$ Tregs, together with their high-level expression of various accessory molecules, may guarantee their prompt and efficient activation upon encounter with a diverse range of self-peptide complex/major histocompatibility complex (MHC) in the periphery, ensuring dominant control of self-reactive T cells.[45]

Many cytokines and chemokines released by immune cells and tumor cells promote angiogenesis and tumor growth.[46]

First discovered as a growth factor for nonimmune cells, TGF-β has gradually been recognized as a critical cytokine in regulating immune responses.[47,41] The consequent overexpression of TGF-β affects the surrounding stromal cells, immune cells, and endothelial cells, thus leading to adverse immunosuppression, angiogenesis, and tumor invasion.[48]

TGF-β exerts systemic immune suppression, inhibits host immunosurveillance, and could significantly regulate immune cell populations.[49] Specifically, TGF-β has been shown to suppress the antitumor activity of T cells, NK cells, neutrophils, monocytes, and macrophages that are known to have a significant role in the regulation of tumor progression.[50,12] Together, these cell populations have the ability to promote or suppress tumor progression depending on the context of each interaction.[51] Coe et al.[52] verified that TGF-β signaling on natural Treg cells is also essential for tumor-induced expansion.

In systemic effects, TGF-β regulates infiltration of inflammatory/immune cells and cancer-associated fibroblasts in the tumor microenvironment, causing direct changes in tumor cells.[53,54]

$CD4^+$ Treg cells can profoundly suppress host immune responses and induce self-tolerance in addition to having an inhibitory effect on cancer immunotherapy.[55,14] According to Langier et al.,[56] although there are still many questions that remain to be answered, the general consensus of the reports in the literature is that Tregs play an important active role in maintaining peripheral immune tolerance. The same authors suggested that Tregs can suppress the immune system by two principal pathways: the direct suppression of the target cells by cell contact and by the secretion of suppressor cytokines. Multiple leukocyte subsets are subjected to Treg cell-mediated suppression.[57] Tregs inhibit the proliferation and effector functions of conventional CD4+ and CD8+ T lymphocytes,[58,59] natural killer T (NKT) cells,[60] B cells,[61] DCs,[62] natural killer (NK) cells,[63] and cells of the monocyte/macrophage lineage.[64]

TGF-β and Regulatory T cells: Implications for Immunopathogenesis of Cancer

Tumor microenvironment is a broad term that refers to all the cells and signaling factors present in any specific cancerous lesion. Matrix and stromal signals are essential for the regulation of cancer, and many of these interactions involving TGF-β have been discussed.[65,66]

TGF-β can act as both a tumor suppressor and as a significant stimulator of tumor progression, invasion, and metastasis.[67] At early stages of tumorigenesis, this cytokine acts directly on the cancer cell to suppress tumor outgrowth. As the tumor progresses, however, genetic and/or biochemical changes allow TGF-β to stimulate tumor progression by its pleiotropic activities on both the cancer cell per se and on nonmalignant stromal cells.[68]

The specific response to TGF-β during tumor progression can be attributed to both independent and interrelated factors, including changes in receptor expression, availability of downstream signaling components, evasion of the immune response, stimulation of inflammation, presence of local and systemic factors (autocrine, endocrine, paracrine, juxtacrine, or matricrine interactions), and the recruitment of cell types that lead to an advantage in tumor growth or promote angiogenesis.[69] Also, the TGF-β pathway has been

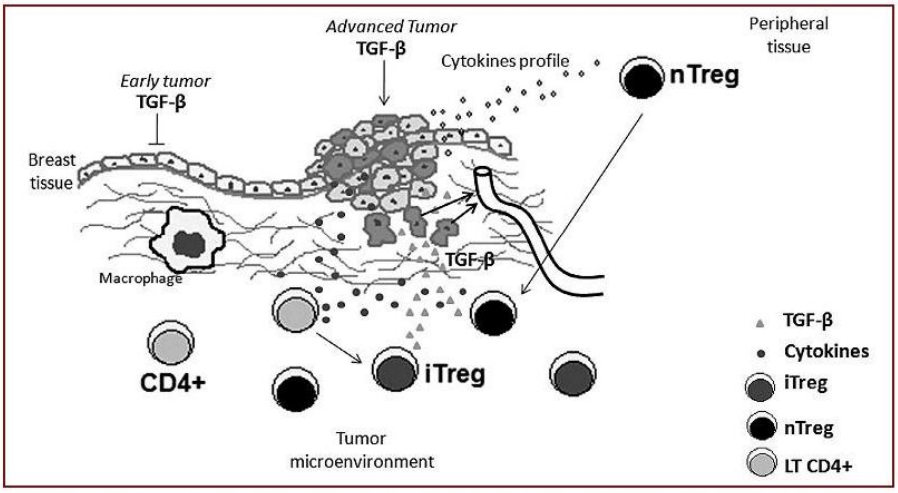

Figure 1. Transforming growth factor β (TGF-β) and regulatory T cells (Treg): the interface of tumor and host immunity in breast cancer. Signals from the microenvironment have a profound influence on the maintenance or progression of malignant tumors. It is known that TGF-β can act as both a tumor suppressor in early stages of disease and as a significant stimulator of tumor progression, invasion, and metastasis in advanced stages. Tregs consist predominantly of $CD4^+$ cells and can be divided into two principal subsets: natural Tregs (nTreg) and adaptative or induced Tregs (iTreg). Cytokines profile from the tumor microenvironment attracts nTreg from the peripheral tissue and induces T $CD4^+$ cell from its own primary tumor to differentiate to iTreg. These cells and the own tumor cell release TGF-β, a cytokine with suppressive characteristic, that acts in the advanced stage of tumor stimulating and favoring the cell invasion and metastasis.

implicated in metastatic processes and has been shown to dramatically impact the ability of tumor cells to spread throughout the body.[68,70]

According to Fahmi et al.,[71] in cancer, the phenotype and/or the function of T cells may differ according to their distribution through immune-associated tissues, namely immune compartments, ie, tumor tissue, tumor-draining (sentinel) lymph nodes, and peripheral blood. In a chemically-induced breast cancer model, these authors observed that the ratio of $CD8^+$ cytotoxic and $CD4^+$ helper T cells from the tumor tissue was not significantly different than other immune compartments. On the other hand, most of these cells were further identified with $CD4^+CD25^{hi}$ or $CD4^+Foxp3^+$, $CD8^+Foxp3^+$ regulatory phenotype. They also observed a selective presence of Tregs in the mammary tumors but not in neighboring mammary tissue, and TGF-β were determined as the major suppressive cytokine secreted by the immune cells of tumor-bearing animals. Hence, they found a differential distribution of T cell subsets through the spleen, mammary lymph nodes, and tumor mass that may contribute to a tumor-associated immunosuppression. Ohara et al.[72] have verified the involvement of regulatory T cells in tumor onset and progression in primary breast cancer.

Some studies have consistently demonstrated the enrichment of $CD4^+$ Tregs in the tumor infiltrating lymphocytes (TILs) in various malignant tumor masses,[73] including lung, breast, pancreas, and ovarian cancers.[74,5] These may also block the activities of tumor-infiltrating immune effectors by recruiting or inducing one or more of the Treg subsets into the tumor microenvironment that has been shown in animal models.[75]

Tregs produce TGF-β, whose pathway encompasses many metastatic processes and that has also been shown to dramatically change the ability of tumor cells to spread throughout the body.[68,76–78]

Vascular endothelial growth factor (VEGF) expression has been shown to be positively correlated with tumor vascularity development and malignancy in various studies. It has been suggested that activation of Tregs releases excessive levels of TGF-β1 that would indirectly induce VEGF expression and subsequent increased vascular development and tumor progression rate. It implies that FoxP3 levels that serve as an indicator of Treg activity may also be contemplated as an indicative of breast tumorigenesis. As invasion, size, and vascularity are prognostic parameters in breast cancer, their positive correlation with FoxP3 expression suggests a progression-marking role of this protein in the transition of breast cancer to an aggressive phenotype.[79]

It is known that signals from the microenvironment have a profound influence on the maintenance or progression of malignant tumors. TGF-β plays an important role in various cell functions, including cell proliferation and differentiation and thus has a crucial function in tumorigenesis process. T cells present the most important immunological response in tumor growth and they become regulatory cells (Tregs) in the presence of appropriate stimulation and interactions with tumor cells (**Figure 1**). Both these immunological effectors act in a coordinated manner, because TGF-β stimulates the development of Tregs and also is a kind of cytokine produced by them and by the tumor cells themselves, a mechanism that has important implications in tumor progression, constituing the subject of much current research.

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ARTIGO 2

Genetic polymorphism in *FOXP3* gene: imbalance in regulatory T-cell role and development of human diseases

REVIEW ARTICLE

Genetic polymorphism in *FOXP3* gene: imbalance in regulatory T-cell role and development of human diseases

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Abstract

The *FOXP3* gene encodes a transcription factor thought to be important for the development and function of regulatory T cells (Treg cells). These cells are involved in the regulation of T cell activation and therefore are essential for normal immune homeostasis. Signals from microenvironment have a profound influence on the maintenance or progression of diseases. Thus, Tregs have an important marker protein, FOXP3, though it does not necessarily confer a Treg phenotype when expressed. *FOXP3* polymorphisms that occur with high frequency in the general populations have been studied in common multifactorial human diseases. Dysfunction of *FOXP3* gene product could result in lack of Treg cells and subsequently chronically activated CD4+ T cells which express increased levels of several activation markers and cytokines, resulting in some autoimmune diseases. In contrast, high Treg levels have been reported in peripheral blood, lymph nodes, and tumour specimens from patients with different types of cancer. The present study discusses the polymorphisms located in intron, exon and promoter regions of *FOXP3* which have already been investigated by many researchers. *FOXP3* has received considerable attention in attempts to understand the molecular aspect of Treg cells. Therefore, in the present study, the relationship between genetic polymorphism of *FOXP3* in Treg-cell role and in disease development are reviewed considering the interactive effect of genetic factors.

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Introduction

The immune system, a highly effective and dynamic cellular network, protects a host from pathogens. Therefore, the immune system should distinguish self from non-self structures, but also between harmful and innocuous foreign antigens (Ags) to prevent nonessential and self-destructive immune responses (Jonuleit and Schmitt 2003).

Regulatory T cells (Tregs) are a unique CD4+ T cell lineage that plays an indispensable role in maintaining immunological unresponsiveness to self-Ags and in suppressing excessive immune responses deleterious to the host. However, they also limit beneficial responses by suppressing sterilizing immunity and limiting antitumour immunity (Sakaguchi *et al.* 2008; Vignali *et al.* 2008; Toker and Huehn 2011).

Most CD4+ cells are commonly characterized by high surface expression of the interleukin 2 (IL-2) receptor α - chain (CD25) and their phenotype is now generally accepted as $CD4^+CD25^{hi}CTLA4^+GITR^+$*FOXP3*$^+$ $CD45RO^+CD45RA^-CD69^-Ki\text{-}67^-$ (Sakaguchi 2005; Betts *et al.* 2006). FOX (forkhead box) is now used as the symbol for all chordate forkhead transcription factors. A phylogenetic analysis has resulted in the definition of 15 classes for all known FOX proteins, so these transcription factors are classified in terms of structure not function. The *FOXP3* (transcription factor forkhead box3) is a member of the forkhead winged-helix transcription-factor family and has three discernible functional domains: a single C2H2 zinc-finger motif (amino acids 200–223), a leucine-zipper-like motif (amino acids 240–261) and a carboxy-terminal forkhead domain (amino acids 338–421). FOXP3 is expressed primarily in a subset of CD4+ T-cells expressing CD25, known as regulatory T cells where it appears to be a key lineage commitment factor for the development of this important subset

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of cells (Coffer and Burgering 2004; Anover *et al.* 2006; Campbell and Ziegler 2007; Eastell *et al.* 2007).

Therefore, FOXP3 has a defining role in regulating the development and function of regulatory T cells. Tone and Greene (2011) and Williams and Rudensky (2007) demonstrated that the continued expression of FOXP3 in mature Treg cells is indispensable for the maintenance of the dominant tolerance that those cells mediate.

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

Caution needs to be exercised when interpreting data on FOXP3 expression in tumours. Increased levels of *FOXP3* mRNA expression may be a result of not only an increased influx of immune cells that express *FOXP3*, like Treg CD4+, Treg CD8+ (Liu *et al.* 2011), macrophages (Leavy 2011; Manrique *et al.* 2011) but also increased expression of *FOXP3* directly in tumour cells. This understanding is importance for developing assays on the basis of FOXP3 for prognosis or drug monitoring. The expression of FOXP3 in tumour cells indicates that FOXP3-targeted drugs must be able to penetrate the tumour bed, which is much more challenging than depleting FOXP3 in the periphery (Lu 2009).

Polymorphisms have been described in various regions of the *FOXP3* gene, such as the promoter, intron and exon regions. According to Hoogendoorn *et al.* (2003), promoters are involved in initiating transcription and are therefore among the many important *cis*-acting elements that regulate gene expression that might harbour functionally relevant polymorphisms.

Polymorphisms of *FOXP3* gene may change its role functionally or quantitatively, therefore leading to lack of functional $CD4^+CD25^+$Tregs, resulting in some autoimmune diseases (Wildin *et al.* 2002), such as immunodysregulation, polyendocrinopathy, enteropathy, X-linked IPEX (immunodysregulation polyendocrinopathy enteropathy) syndrome (van der Vliet and Nieuwenhuis 2007), type 1 diabetes (T1D) (Bassuny *et al.* 2003) and autoimmune thyroid diseases (Ban *et al.* 2007).

In this context, *FOXP3* polymorphisms that occur with high frequency in the general population have been studied in common multifactorial human diseases, and some of these studies are discussed in this review.

Forkhead box 3 (FOXP3)

Most transcriptional factors are modular proteins composed of DNA-binding domains and/or motifs that interact with other transcriptional regulators and modifying enzymes. Many of these interacting proteins do not bind to DNA directly, but modulate DNA binding by conferring transcriptional activating or repressing activity to the DNA binding-partner. This activity is often related to either compaction or relaxation of chromatin, thus restricting or permitting access of other transcriptional regulatory proteins (Li *et al.* 2004).

FOXP3 gene was identified in 2001 as the disease-causative gene in Scurfy mice, which spontaneously develop severe autoimmunity/inflammation as a result of a single-gene mutation on the X chromosome (Brunkow *et al.* 2001). The human full-length *FOXP3* gene is 1296 bp in size and has been reported to consist of 11 different exons. This gene is located at the small arm of the X-chromosome (Xp11.23), that is subject to X-chromosomal inactivation, and encodes a 431 amino-acid protein (Gambineri *et al.* 2003; Fontenot *et al.* 2005; Ban *et al.* 2007; Torgerson and Ochs 2007). Sequence analyses have revealed three highly conserved noncoding regions in *FOXP3* locus, all of which have been found to be subject to epigenetic modifications and involved in regulating the transcription of *FOXP3* (Huehn *et al.* 2009).

The first highly conserved region is the *FOXP3* promoter, which is located 6.5-kb upstream of the first coding exon of *FOXP3*. This promoter is a classic TATA and CAAT-box-containing sequence that is activated in response to TCR signalling through binding of NFAT (nuclear factor of activated T lymphocytes) and AP1 (activator protein 1) (Mantel *et al.* 2006). The second highly conserved noncoding region in the *FOXP3* locus has been identified as a TGFβ-sensitive element that contains binding sites for NFAT and SMADs (Tone *et al.* 2008). The most striking differences regarding the methylation pattern of the *FOXP3* locus have been observed in a third, highly conserved, CpG-rich enhancer and/or stabilizer region. This site was found to be fully demethylated in Treg cells and methylated in conventional T cells (Baron *et al.* 2007; Floess *et al.* 2007; Kim and Leonard 2007; Nagar *et al.* 2008), and normally referred as the Treg-cell-specific demethylated region (TSDR).

The three functional domains described above, single C2H2 zinc-finger motif, a leucine-zipper-like motif and a carboxy-terminal forkhead domain, are involved in DNA binding, nuclear transport (Hancock and Ozkaynak 2009), homomeric and heteromeric complex formation (Li *et al.* 2006), and transcriptional repressor activity (Li *et al.* 2006; Lopes *et al.* 2006). FOXP3 interacts with multiple transcription factors known to be involved in activation, differentiation, and response of CD4+ T cells to TCR stimulation, NFAT), nuclear factor—kappa B (NF-kB), runt-related transcription factor 1 (RUNX1), retinoic acid receptor-related orphan receptors (RORs) (RORα and RORγT), IFN regulatory factor 4 (IRF4), signal transducer and activator of transcription 3 (STAT3), and Jun (Bettelli *et al.* 2005; Wu *et al.* 2006; Ono *et al.* 2007; Du *et al.* 2008; Zhou *et al.* 2008; Chaudhry *et al.* 2009; Rudra *et al.* 2009; Zheng *et al.* 2009).

Genomewide analysis has shown that FOXP3 binds to the promoter region of 700–1100 bp of some genes, many of

those being associated with TCR signalling. A large number of FOXP3-bound genes were upregulated or downregulated in FOXP3+ T cells, indicating that FOXP3 may act as both a transcriptional activator and repressor (Marson *et al.* 2007; Zheng *et al.* 2007). Chromatin immunoprecipitation combined with microarray analyses revealed numerous transcriptional targets of human and murine FOXP3, including genes whose expression is upregulated (*CD25* (cluster of differentiation 25), *CTLA4* (cytotoxic T-lymphocyte antigen 4), *TNFRSF18* (GITR, glucocorticoid-induced TNFR family related gene) or repressed (*IL2* (interleucin-2), *PTPN22* (protein tyrosine phosphatase, nonreceptor type 22)) (Marson *et al.* 2007; Zheng *et al.* 2007).

FOXP3 cooperates with additional transcription factors, such as NFAT1 and RUNX1, to regulate gene expression (Wu *et al.* 2006; Ono *et al.* 2007; Kitoh *et al.* 2009; Rudra *et al.* 2009). Transcriptional repression by FOXP3 may reflect its ability to recruit histone deacetylase (HDAC) family members through an N-terminal repressor domain (Li and Greene 2007; Li *et al.* 2007a). However, the same N-terminal domain is also responsible for transcriptional activation by FOXP3 (Wu *et al.* 2006), and the stimulatory effects of FOXP3 on gene expression cannot be explained by recruitment of HDAC corepressor complexes alone.

The C-terminal forkhead domain, where the largest number of IPEX missense mutations cluster, is required for DNA binding and nuclear import. The leucine zipper of *FOXP3* is necessary and sufficient to mediate both homo-association (Chae *et al.* 2006; Lopes *et al.* 2006; Li *et al.* 2007b) and hetero-association with FOXP1 (Wang *et al.* 2003). Although the forkhead domain alone may bind to DNA *in vitro* (Stroud *et al.* 2006; Bandukwala *et al.* 2011), the disease-associated mutations of the leucine zipper domain disrupting *FOXP3* dimerization can substantially reduce the binding of FOXP3 to promoter regions *in vivo* (Chae *et al.* 2006; Lopes *et al.* 2006; Li *et al.* 2007b). Moreover, the leucine zipper is also important for the interaction between histone H1.5 and FOXP3, which cooperatively repress IL-2 transcription in human T cells (Mackey-Cushman *et al.* 2011). A repression domain in the N-terminal proline-rich region is required for FOXP3 to suppress transcription (Lopes *et al.* 2006).

Genetic polymorphism in *FOXP3* gene: promoter region

Genetic polymorphisms of *FOXP3* in the promoter region have been widely studied in the context of autoimmune diseases (table 1).

Mutations in the open-reading frame of *FOXP3* are associated with IPEX, a rare fatal paediatric condition (Bennett *et al.* 2001), exhibiting aggressive autoimmune features. *FOXP3* mutations in IPEX patients result in heterogeneous biological abnormalities, leading not necessarily to a lack of differentiation of $CD4^+CD25^{high}$Tregs but rather to a dysfunction in these cells and in effector T cells (Bacchetta *et al.* 2006). Thus, polymorphisms of the *FOXP3* gene may change FOXP3 functionally or quantitatively, thus leading to the lack of functional $CD4^+CD25^+$Tregs and subsequently chronically activated $CD4^+$ T cells which express increased levels of several activation markers and cytokines, resulting in autoimmune diseases (Wildin *et al.* 2002; Bjornvold *et al.* 2006).

Polymorphisms in the promoter region may potentially alter gene expression by changing the binding specificity of transcription factors to their binding sites and by modifying the kinetics of transcription initiation (Hanel *et al.* 2011). There are five single-nucleotide polymorphisms (SNP) in the promoter region of *FOXP3*: −924A/G (rs2232365), −1383C/T (rs2232364), −2383C/T (rs3761549), −3279C/A (rs3761548) and −3499A/G (rs3761547) (Bassuny *et al.* 2003).

The *AA* genotype of the −3279C/A polymorphism causes the loss of binding with some transcription factors, such as E47 and C-Myb, leading to defective transcription of *FOXP3*. Moreover, the *A* allele of this polymorphism is associated with a dramatic reduction in luciferase activity compared with the *C* allele (Shen *et al.* 2010). Conversely, there is no report on the functional effects of −2383C/T and −3499A/G polymorphisms on gene expression (Inoue *et al.* 2010).

The −3279 (rs3761548) polymorphism has been largely studied and various associations with diseases have been described. Gao *et al.* (2010) evaluated the susceptibility to psoriasis in a Han Chinese population. These authors found an increased risk for psoriasis associated with the *AC* genotype and also to the combined *AC* + *AA* genotypes compared with the wild *CC* genotype. An association between the −3279 polymorphism and allergic rhinitis (AR) in heterozygous form was also identified in this population, especially in response to house dust mite (Zhang *et al.* 2009). Fodor *et al.* (2011) confirmed the findings of Zhang *et al.* (2009) when they examined whether the association detected in the Chinese population also exists in a European population of Hungarian Caucasian patients with ragweed pollen allergy. According to this study, females homozygous for the rare *FOXP3* rs3761548 allele (*AA*) are protected against AR; otherwise, females who are either wild-type (*CC*) or heterozygote carriers (*CA*) of the rare allele are more susceptible to AR. Bottema *et al.* (2010) also found that in females, the −3279 (rs3761548) was significantly associated with sensitization to egg at age one and two years, and with sensitization to indoor allergens at age two, but not at age four and eight years.

The −3279 (rs3761548) polymorphism in the *FOXP3* gene was also associated with the development and intractability of Graves' disease (GD) (Inoue *et al.* 2010), with lower anti-dsDNA levels in female systemic lupus erythematosus patients (Lin *et al.* 2011), and also significantly associated with unexplained recurrent spontaneous abortion (URSA) in the Chinese Han population (Wu *et al.* 2012).

Table 1. *FOXP3* polymorphisms in promoter region.

Polymorphism	Diseases	Year	Country	Reference
rs2232365	Crohn's disease	2005	USA	Park *et al.* (2005)
	Graves' and Addison's disease	2006	UK	Owen *et al.* (2006)
	Psoriasis	2010	China	Gao *et al.* (2010)
	Unexplained recurrent spontaneous abortion	2012	China	Wu *et al.* (2012)
rs3060515	Allergic rhinitis	2009	China	Zhang *et al.* (2009)
	Systemic lupus erythematosus	2011	Taiwan	Lin *et al.* (2011)
rs3761547	Juvenile idiopathic arthritis	2007	UK	Eastell *et al.* (2007)
	Allergic rhinitis	2009	China	Zhang *et al.* (2009)
	Hashimoto's disease and Graves' disease	2010	Japan	Inoue *et al.* (2010)
rs3761548	Crohn's disease	2005	USA	Park *et al.* (2005)
	Breast cancer	2009	Israel	Raskin *et al.* (2009)
	Allergic rhinitis	2009	China	Zhang *et al.* (2009)
	Atopy	2010	The Netherlands	Bottema *et al.* (2010)
	Psoriasis	2010	China	Gao *et al.* (2010)
	Hashimoto's disease and Graves' disease	2010	Japan	Inoue *et al.* (2010)
	Endometriosis and infertility	2011	Brazil	Andre *et al.* (2011)
	Allergic rhinitis	2011	Hungary	Fodor *et al.* (2011)
	Systemic lupus erythematosus	2011	Taiwan	Lin *et al.* (2011)
	Unexplained recurrent spontaneous abortion	2012	China	Wu *et al.* (2012)
rs3761549	Graves' and Addison's disease	2006	UK	Owen *et al.* (2006)
	Hashimoto's disease and Graves' disease	2010	Japan	Inoue *et al.* (2010)
	Endometriosis and infertility	2011	Brazil	Andre *et al.* (2011)
rs5902434	Psoriasis	2010	China	Gao *et al.* (2010)
	Unexplained recurrent spontaneous abortion	2012	China	Wu *et al.* (2012)
(GT)*n*	Type 1 diabetes	2003	Japan	Bassuny *et al.* (2003)
	Type 1 diabetes	2004	Italy	Zavattari *et al.* (2004)
	Systemic lupus erythematosus, rheumatoid arthritis, ulcerative colitis, Crohn's disease and celiac disease	2005	Spain	Sanchez *et al.* (2005)
	Type 1 diabetes and coeliac disease	2006	Norway	Bjornvold *et al.* (2006)
	Graves' and Addison's disease	2006	UK	Owen *et al.* (2006)
	Autoimmune thyroid diseases	2007	Japan	Ban *et al.* (2007)
	Type 1 diabetes	2007	Japan	Nakanishi and Shima (2007)

Although several studies have found associations between −3279 (rs3761548) polymorphism and diseases, this is not always the case in all studies. Andre *et al.* (2011) found no association with endometriosis related infertility group or the idiopathic infertility group. There was also no association between this polymorphism with Crohn's disease (Park *et al.* 2005) and breast cancer risk (Raskin *et al.* 2009).

Various studies with $(GT)_n$ microsatellite polymorphisms in the promoter region of *FOXP3* gene have been conducted, particularly examining association with T1D, but with varying results. Bassuny *et al.* (2003) demonstrated that the $(GT)_{15}$ allele showed a significant higher frequency in patients with T1D than in controls. According to these results, the authors concluded that the *FOXP3*/*scurfin* gene appears to confer a significant susceptibility to T1D in the Japanese population. This association, however, was not found by Nakanishi and Shima (2007) who also studied microsatellite polymorphism in the promoter region of *FOXP3* gene in the Japanese population. Further, attempts to confirm this association in independent cohorts of different ethnic origin and other autoimmune diseases have been unsuccessful. Using 418 T1D families and a further 268 male patients and 326 healthy males for a case–control analysis from Sardinia, Zavattari *et al.* (2004) detected no association between the polymorphisms and T1D, nor with seven SNPs and four other microsatellites in the *FOXP3* gene region. This conclusion was also obtained by Bjornvold *et al.* (2006) who analysed the same microsatellite analysed by Bassuny *et al.* (2003) in a Caucasian population. Howson *et al.* (2009) tested six SNPs in *FOXP3* for association with T1D, among these the rs4824747, but found no evidence of association.

The $(GT)_n$ microsatellite polymorphism in *FOXP3* was also investigated in other autoimmune diseases, like the work of Sanchez *et al.* (2005) that investigated this polymorphism in relation with systemic lupus erythematosus, rheumatoid arthritis, ulcerative colitis, Crohn's disease and celiac disease. They found no evidence of association between this genetic variant with autoimmune diseases in a Spanish population. Ban *et al.* (2007) studied autoimmune thyroid diseases and found that the $(GT)_n$ microsatellite polymorphism is associated with these diseases in a Caucasian cohort but not in a Japanese cohort. Also, no evidence was found that these $(GT)_n$ microsatellite polymorphism contributed to susceptibility to Graves' or Addison's diseases in the northeast England population (Owen *et al.* 2006).

The −924 (rs2232365) SNP is significantly associated with unexplained recurrent spontaneous abortion in the Chinese Han population, highlighting the important role of *FOXP3* in successful pregnancy (Wu *et al.* 2012). But no associations were found between this SNP with susceptibility to psoriasis in a Han Chinese population (Gao *et al.* 2010), Crohn's disease (Park *et al.* 2005) and Graves' disease (Owen *et al.* 2006).

Lin *et al.* (2011) found an evidence for association of the −6054 (rs3060515) SNP with lower risk of lupus nephritis. This SNP was also related to AR, whose disease is also associated with the −3499 (rs3761547) polymorphisms (Zhang *et al.* 2009). However the −3499 (rs761547), was not related to juvenile idiopathic arthritis (JIA) (Eastell *et al.* 2007). This polymorphism also was genotyped by Inoue *et al.* (2010) in Japanese patients to clarify their effects on the development and prognosis of autoimmune thyroid diseases, such as Hashimoto's disease (HD) and Graves' disease. The authors found no difference in genotypes and allele frequencies among the groups. In this same work the authors genotyped −2383 (rs3761549) polymorphism in *FOXP3* gene, which was related to severity of HD. The −3499 (rs3761549) have also significant association with endometriosis, regardless of the stage of the disease (Andre *et al.* 2011). However, this SNP was not associated with the susceptibility to Graves' disease or Addison's disease (Owen *et al.* 2006).

The −6054 (rs5902434) SNP was evaluated in relation to psoriasis in a Han Chinese population by Gao *et al.* (2010) and no association was found, but the evaluation of −6054 (rs5902434) SNP with respect to URSA by Wu *et al.* (2012) showed that this polymorphism of the *FOXP3* gene may confer an important susceptibility in Chinese population.

Genetic polymorphism in *FOXP3* gene: intron region

Some mutations in *FOXP3* gene are localized in intron region, as shown in table 2. Andre *et al.* (2011) analysed three SNPs in intron regions of *FOXP3* gene: −20 (rs2232368) located in the intron 1, +87 (rs2232366) located in intron 5 and +459 (rs2280883) located in intron 9. Their results revealed a possible association of +459 (rs2280883) and −20 (rs2232368) with idiopathic infertility whereas no association was found for +87 (rs2232366) either for the endometriosis-related infertility group or the idiopathic infertility group. When Park *et al.* (2005) investigated the SNP +459 (rs2280883) located in intron 9 of *FOXP3* they observed a significant difference between patients with primary billiary cirrhosis (PBC) and controls. This SNP is also associated with severe psoriasis patients (Gao *et al.* 2010), but not related to susceptibility to Graves' and Addison's

Table 2. *FOXP3* polymorphisms in intron region.

Polymorphism	Disease	Year	Country	Reference
rs2232366	Endometriosis and infertility	2011	Brazil	Andre *et al.* (2011)
rs2232367	Juvenile idiopathic arthritis	2007	UK	Eastell *et al.* (2007)
rs2232368	Endometriosis and infertility	2011	Brazil	Andre *et al.* (2011)
rs2280883	Crohn's disease	2005	USA	Park *et al.* (2005)
	Graves' and Addison's disease	2006	UK	Owen *et al.* (2006)
	Juvenile idiopathic arthritis	2007	UK	Eastell *et al.* (2007)
	Psoriasis	2010	China	Gao *et al.* (2010)
	Endometriosis and infertility	2011	Brazil	Andre *et al.* (2011)
rs2294019	Atopy	2010	The Netherlands	Bottema *et al.* (2010)
rs2294020	Juvenile idiopathic arthritis	2007	UK	Eastell *et al.* (2007)
	Breast cancer	2009	Israel	Raskin *et al.* (2009)
rs2294021	Graves' and Addison's disease	2006	UK	Owen *et al.* (2006)
	Atopy	2010	The Netherlands	Bottema *et al.* (2010)
	Unexplained recurrent spontaneous abortion	2012	China	Wu *et al.* (2012)
rs4824747	Juvenile idiopathic arthritis	2007	UK	Eastell *et al.* (2007)
	Type 1 diabetes	2010	UK	Howson *et al.* (2009)
rs5906761	Breast cancer	2009	Israel	Raskin *et al.* (2009)
	Atopy	2010	The Netherlands	Bottema *et al.* (2010)
rs6609857	Graves' and Addison's disease	2006	UK	Owen *et al.* (2006)
	Juvenile idiopathic arthritis	2007	UK	Eastell *et al.* (2007)
	Atopy	2010	The Netherlands	Bottema *et al.* (2010)
	IPEX syndrome	2001	USA	Bennett *et al.* (2001)
$(TC)_n$	Type 1 diabetes	2003	Japan	Bassuny *et al.* (2003)
	Graves' and Addison's disease	2006	UK	Owen *et al.* (2006)
	Type 1 diabetes	2004	Italy	Zavattari *et al.* (2004)
	Type 1 diabetes and coeliac disease	2006	Norway	Bjornvold *et al.* (2006)
	Autoimmune thyroid diseases	2007	Japan	Ban *et al.* (2007)

Table 3. *FOXP3* polymorphisms in exon region.

Exon	Disease	Year	Country	Reference
1	IPEX syndrome	2006	Italy/France/USA	Anover *et al.* (2006)
12	Autoimmune disease and the risk of FVIII inhibitor development	2010	Italy	Bafunno *et al.* (2010)
2–12	Acute leukemias	2011	Korea	Kim *et al.* (2011)

diseases (Owen *et al.* 2006) and juvenile idiopathic arthritis (Eastell *et al.* 2007).

Located in intron 5 of the *FOXP3* gene, there is also a $(TC)_n$ microsatellite polymorphism. This was analysed by Ban *et al.* (2007), showing an association with autoimmune thyroid disease in a Caucasian cohort, especially in male patients, however this association was not seen in a Japanese cohort. The $(TC)_n$ microsatellite polymorphism also was not related to Graves' and Adisson's diseases (Owen *et al.* 2006) and T1D (Bassuny *et al.* 2003; Zavattari *et al.* 2004; Bjornvold *et al.* 2006).

Bottema *et al.* (2010) studied four intron regions of *FOXP3* gene: rs5906761 (located upstream of exon −1) and rs2294021, rs2294019 and rs6609857 (located in 3'UTR) and observed significant association between these SNPs with sensitization, in girls, to egg at age one and two years, and with sensitization to indoor allergies at age two, but not at four and eight years. They also found that rs5906761 and rs2294021 were associated with remission of sensitization to food allergens in boys. The rs5906761 was also studied by Raskin *et al.* (2009), but in their work no association was found between this polymorphism and breast cancer risk. The rs2294020 SNP was also not associated with juvenile idiopathic arthritis (Eastell *et al.* 2007).

Of the SNPs located in 3'UTR of *FOXP3* gene, two of them were also investigated by Owen *et al.* (2006): the rs2294021 and rs6609857, but the authors did not find any evidence that these contribute to Graves' or Addison's disease susceptibility in northeast England population. The polymorphism rs2294021 was not related with recurrent spontaneous abortion (Wu *et al.* 2012) and rs6609857 was not associated with JIA (Eastell *et al.* 2007). The same authors studied the rs2232367 and rs4824747, such as the other SNPs, in relation to JIA, and also found no associations.

Bennett *et al.* (2001) identified an A→G transition within the first polyadenylation signal (AAUAAA→AAUGAA), which resides 878–883 bp downstream of the stop codon. This mutation was not detected in over 212 normal individuals, suggesting that it is causal of IPEX by a mechanism of nonspecific degradation of the *FOXP3* gene message.

Genetic polymorphism in *FOXP3* gene: exon region

There are few studies involving the expression control and modulation in the exon region of *FOXP3* gene (table 3). Anover *et al.* (2006) showed a novel 1388 bp deletion mutation that included the 5' half exon −1 (noncoding) and a large segment of the first intron on *FOXP3* gene. This aberrantly spliced mRNA species is rapidly degraded leading to overall low mRNA abundance in the patients and subsequent lack of FOXP3+ Treg, resulting in IPEX.

Kim *et al.* (2011) analysed coding region (exons 2–12) of human *FOXP3* gene in acute leukaemia, but no mutations were detected, indicating that *FOXP3* gene mutations may be specific to just for few cancers, such as breast and prostate cancers. Polymorphisms in exon 12 were also studied by Bafunno *et al.* (2010), whose work revealed no positive or negative association between this polymorphism and the susceptibility to haemophilia A treatment.

Conclusion

The *FOXP3* gene is a central molecule in the function of Tregs cells, both in the context of maintenance of immune tolerance and also in regulation of response. Therefore, this transcription factor is very important to play a crucial role in generation of Treg phenotype. In both autoimmune diseases and cancer, FOXP3 may play a role in immunopathology, due to potent suppressive T-cell activation and effector function (Watanabe *et al.* 2010). Polymorphisms in *FOXP3* gene can lead to immune system imbalance and mediate clinically serious human disease development. The study of these polymorphisms may contribute to a better understanding of their pathogenesis and progression.

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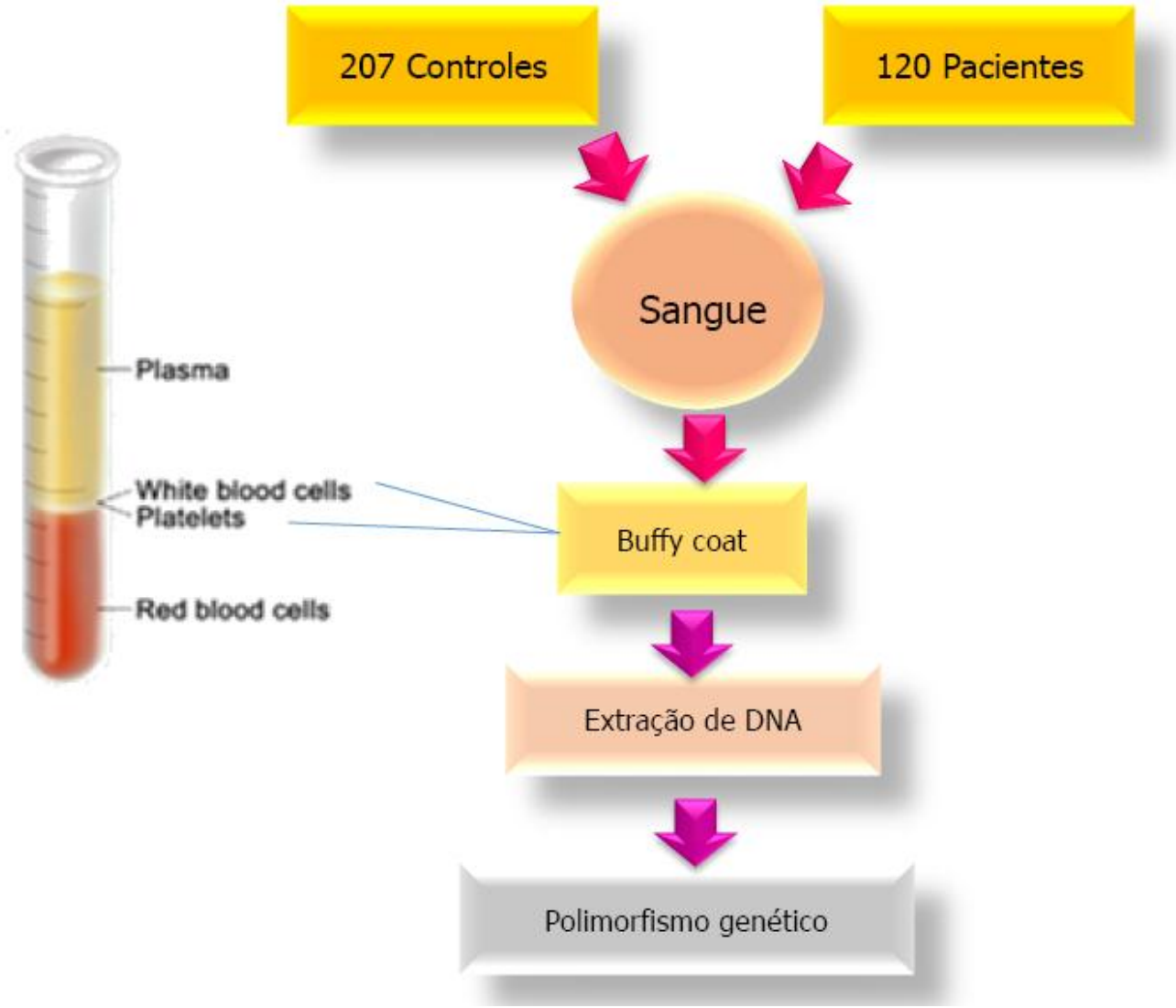
ARTIGO 3

FOXP3 and *TGFB1* genetic polymorphisms as susceptibility markers for human breast cancer

Delineamento Experimental I



RESEARCH ARTICLE

FOXP3 and *TGFB1* genetic polymorphisms as susceptibility markers for human breast cancer

Abstract

Breast cancer is a heterogeneous disease and normally is associated with immune cell infiltration. Regulatory T cells (Tregs), act promoting tumor growth and progress by inhibiting the immune response against cancer. Transforming growth factor B1 (*TGFB1*) is a key molecule for generation and function of Tregs. For these reason, our aim was to investigate the influence of polymorphisms in *FOXP3* (A/G rs2232365 and C/A rs3761548) and *TGFB*1 (T/C rs1800470) in relation to breast cancer susceptibility and clinical outcome. Peripheral blood was obtained from 120 patients and 207 controls, neoplasia free, and all DNAs were amplified by allele specific PCR. The case-control study indicated a positive association for *TGFB1* CC genotype [OR= 1.88; 95% CI (1.19-2.96)] (p=0.0064) and for C allele carriers [OR= 4.41; 95%CI (1.81-10.76)] (p=0.0005), as well as for *FOXP3* allelic variants (G + A) in a combined analysis [OR= 6.18; 95% CI (1.67-22.93)] (p=0.0021) in relation to breast cancer susceptibility. For the clinical features (hormonal receptor, tumor size, lymph node metastasis, HER-2 and clinical staging) of patients, no significant differences in any of the proposed polymorphisms were observed. Our study seems to be the first one that made this kind of analysis and we suggest that these genes can act as susceptibility marker in breast tumorigenesis, which must be confirmed in larger samples.

Key words: breast cancer, *FOXP3*, *TGFB1*, genetic polymorphisms, clinical outcome.

Introduction

Breast cancer is the leading cause of cancer death among women worldwide. Data from the National Cancer Institute revealed that are expected in Brazil 57,120 new cases of breast cancer. It is worth noting that, regardless the type of non-melanoma cancer, the mammary tumor is the most common among women in the Southeast, South, Midwest and Northeast regions of Brazil and accounts for a high morbidity and mortality rates (BRAZIL, 2013).

This neoplasia is a heterogeneous disease with different known biological subclasses (CALLAGY *et al.*, 2003) some of which are associated with immune cell infiltration (HRUBISKO *et al.*, 2010). Normally, inflammation serves to protect an infected or damaged tissue by recruiting cells necessary to resolve the insult and, to restore normal tissue function. However, inflammation sometimes fails to subside which can promote tumor cell growth, survival and angiogenesis (GRIVENNIKOV *et al.*, 2010).

Host immunity plays an important and complex role in regulation of tumor progression (DE VISSER *et al.*, 2006) and the presence of tumor-infiltrating leukocytes at the time of initial diagnosis is believed to reflect the host anti-tumor immune response. Breast cancers are often immunogenic and drive antigen-specific lymphocyte responses (NZULA *et al.*, 2003; BECKHOVE *et al.*, 2004; SOMMERFELDT *et al.*, 2006; CHAPMAN *et al.*, 2007). A subpopulation of $CD4^+$ T cells expressing CD25 and the master transcription factor FOXP3 ($CD4^+CD25^+FOXP3^+$), termed regulatory T cells (Tregs), are involved in promoting tumor growth and progress by inhibiting the immune response against cancer (CHEN *et al.*, 2003; CURIEL *et al.*, 2004; WEI *et al.*, 2006).

One such gene of interest in the function of Tregs is the *FOXP3* that belongs to a family of transcription factors with known function in various cellular processes. The human *FOXP3* contains 11 coding exons and is mapped in the Xp 11.23 chromosome (COFFER; BURGERING, 2004). Polymorphisms in the promoter region of this gene (-/ATT rs3060515, C/A rs3761548, C/T rs3761549 and A/G rs2232365) are associated with some diseases, such as systemic lupus erythematosus (SLE) and primary biliary cirrhosis (PBC) (PARK *et al.*, 2005; LAN *et al.*, 2010), as well as in breast cancer (JAHAN, RAMACHANDER, *et al.*, 2013).

Transforming growth factor beta 1 (*TGFB1*) is another gene that regulates the generation and function of $CD4^+$ $CD25^+$Tregs (CHEN *et al.*, 2003). However, the ultimate effect of TGFB on immune cells is heavily dependent on the microenvironment and on the

presence of other cytokines and factors (CHEN *et al.*, 2006). Also, according to KUO *et al.* (2012), TGFB signaling of immune/inflammatory regulation may be essential for distant metastatic in breast cancer. A functional single nucleotide polymorphism (SNP) in the *TGFB1* is the +869 T/C rs1800470 (Leu10Pro), that modify critical elements in protein sequence (DUNNING *et al.*, 2003; LOKTIONOV, 2004), influencing an increase in production of this cytokine.

Considering that TGFB is important to initiate the expression of FOXP3 and considering that both genes are linked to the expression of regulatory T cells, which promote tumor growth, our study aimed to investigate polymorphisms in these genes (*FOXP3* A/G rs2232365, A/C rs3761548 and *TGFB1* T/C rs1800470) and their relation to breast cancer and clinical outcome of patients.

Materials and Methods

Human Subjects and samples

Following approval from the Human Ethics Committee of the State University of Londrina (CEP/UEL No 233/09), which is in accordance with the National Committee for Ethics in Research (CONEP-CAAE - 0179.0.268-09) and Resolution 196/96 - National Health Council (CNS), peripheral blood was collected from breast cancer patients and healthy blood donors. A Term of Free Informed Consent was signed by all sample donors prior to blood collection. Clinical staging was determined according to the Union of International Control of Cancer classification criteria (UICC; STATISTICS, 2002).

Peripheral blood was drawn in sterile syringes containing heparin, as anticoagulant, from 120 patients with a histopathological diagnosis of breast carcinoma, according to World Health Organization (1993) classification system. Patients were addressed in Londrina Cancer Hospital, Parana State, Brazil. Samples from 207 healthy blood donors, neoplasia free to date, were also obtained from Clinical Hospital from State University of Londrina. Demographic characteristics were controlled between groups (patients and controls) since they were collected at the same region.

DNA extraction

Genomic DNA was extracted from 200uL of peripheral blood from all participants by the BioPur specific Kit (Biometrix Diagnostica, Curitiba, Paraná, Brazil) according to manufacturer's instructions. The DNA samples were quantified by Thermo Fisher Scientific NanoDrop 2000c®Spectrophotometer (USA) at a wavelength of 260nm and 280nm.

FOXP3 and TGFB1genetic polymorphisms

DNA (100ng) was amplified by allelic specific polymerase chain reactions (AS-PCR) using specific primers for *FOXP3* gene (A/G, rs2232365; C/A, rs3761548) according to GAO *et al.* (2010) modified, which the cycles condition where reduced and for *TGFB1*(T/C rs1800470), according to LEE *et al.* (2005) modified, which reagents ($MgCl_2$) conditions were increased to allele T following the GenBank accession numbers NT_079573.4 and NG_013364.1, respectively (Table 1). All reactions were performed with positive and negative controls, to ensure a correct amplification and no contaminations. Amplicons of *TGFB1* and *FOXP3* were analyzed by electrophoresis on acrylamide gel (10 %) and detected by a non radioisotopic technique using a commercially available silver staining method.

Table 1 . Allelic variants and amplicons of *FOXP3* and *TGFB* genes.

GENE	ALLELE	AMPLICON
FOXP3		
-924 (A/G, rs2232365)	(A)	442bp
	(G)	427bp
-3279 (C/A, rs3761548)	(C)	333bp
	(A)	334bp
TGFB		
+869 (T/C, rs1800470)	(T)	297bp
	(C)	204bp

Statistical Analysis

The gene frequencies observed in patients and controls were compared using contingency tables to calculate the Odds Ratios (OR) with a confidence interval (CI) of 95%, in an association study. For these genes in which the three genotypes were identified, a 2x2 contingency table was constructed, with the considered wild type genotype as reference (OR=1.0), to determine the OR value, using the DPP Braile Biomedical program (http://www.braile.com.br). The statistical analyses for clinical and histopathological parameters were realized by Spearman rho correlation two-tailed test, using the SPSS Statistics 17.0 software (SPSS inc., Chicago, Illinois, USA). A p value ≤ 0.05 was considered statistically significant.

Results

In the present study, the age range of 120 women breast cancer patients was 26 – 86 years old and the median age was 54 years. The average size of tumors was 0.6-20 cm (3.07±3.04 cm). Most samples were diagnosed as ductal carcinoma (97.30%), stages II (41.44%) or III (31.1%). In our sample, 73.3% of the patients expressed the estrogen receptor and 72.10% are HER-2 negative.

The electrophoretic profiles according to genotypes of *FOXP3* and *TGFB* are demonstrated in Figure 1.

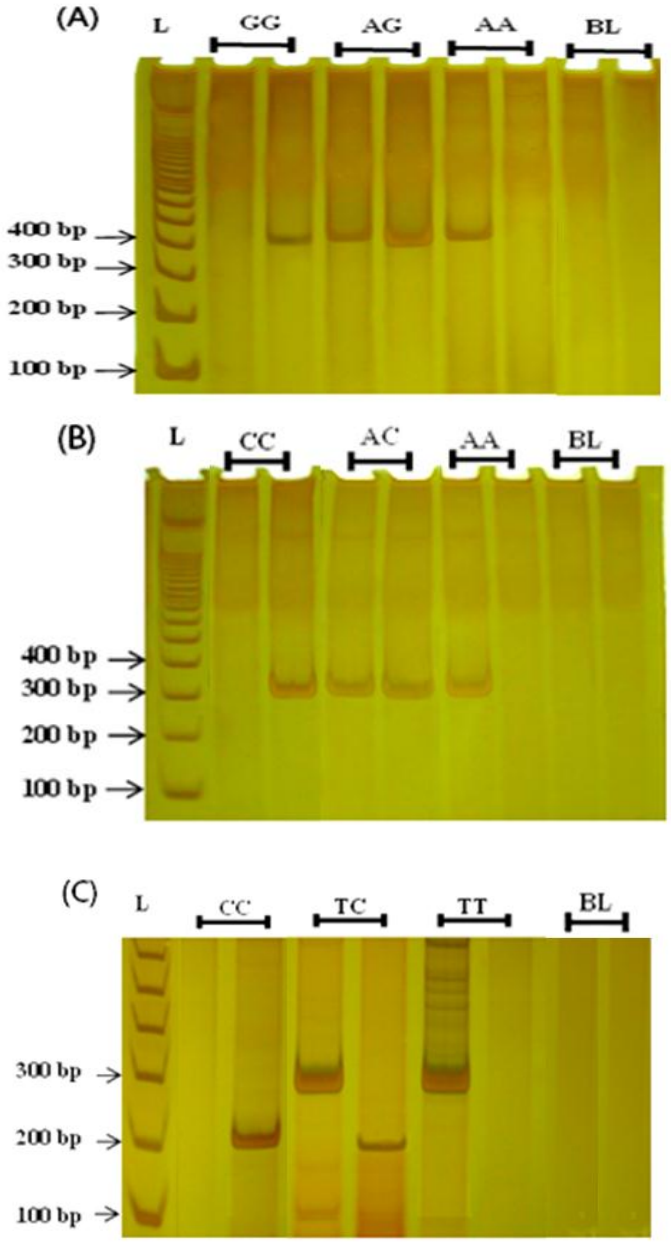


Figure 1. Eletrophoretic profiles for the three polymorphisms analyzed. (A) *FOXP3*A/G rs2232365 shows a fragment of 442bp for allele A and 427bp for allele G. (B) *FOXP3*C/A

rs3761548 shows a band of 334bp for allele A and 333bp for allele C. (C) *TGFB1* T/C rs1800470 which demonstrates a band of 297bp for allele T and 204bp for allele C. BL correspond a negative control and L correspond a 100bp ladder (Invitrogen).

The genotypes distributions of *FOXP3* and *TGFB1* polymorphisms in cases and controls are shown in Figure 2.

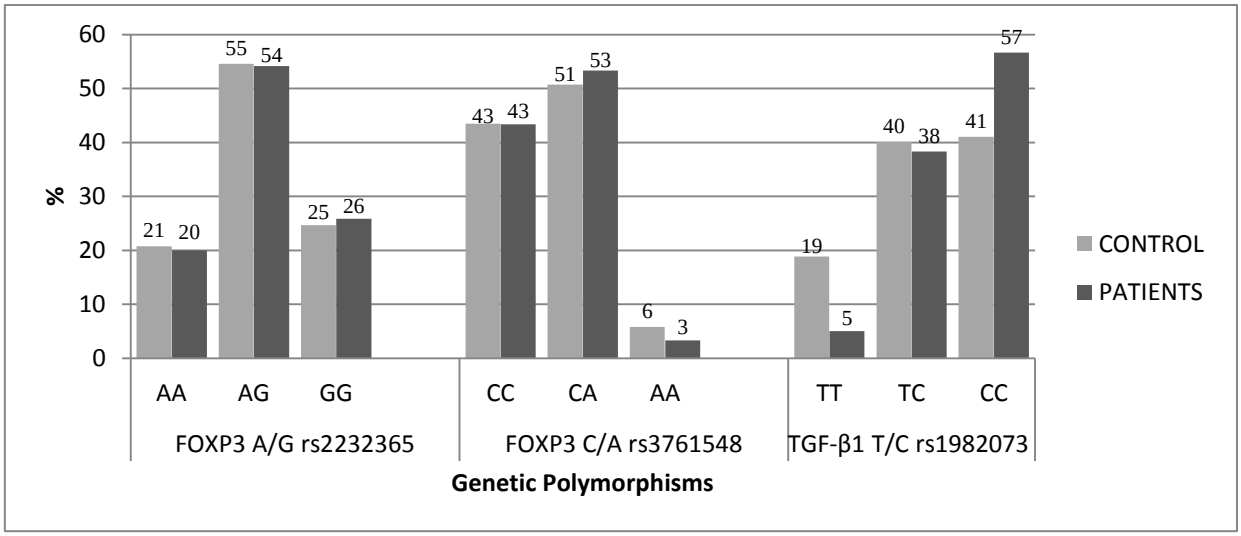


Figure 2. Genotype distribution of *FOXP3* and *TGFB1* polymorphisms in patients and controls. The analysis of the Hardy-Weinberg Equilibrium for the polymorphisms FOXP3A/G rs2232365 and TGFB1 T/C rs18004 showed that the samples are in equilibrium ($p>0.05$), but for the polymorphisms FOXP3C/A rs3761548 the samples are not ($p<0.005$).

Allelic variants were analyzed separately or combined in search for an association with breast cancer susceptibility. The case control study indicated a positive association for *TGFB1* CC homozygous [OR= 1.88 95% CI (1.19-2.96)] (p=0.0064) and C allele carriers [OR= 4.41 95% CI (1.81-10.76)] (p=0.0005). No significant associations were found to *FOXP3* polymorphisms when homozygous genotypes were analyzed: GG [OR= 1.07 95% CI (0.64-1.79)] (p=0.8100) and AA [OR= 0.56 95% CI (0.18-1.78)] (p=0.3195). Likewise, we did not find association for G allele carriers of FOXP3 A/G rs2232365 [OR= 1.05 95% CI (0.60-1.83)] (p=0.8674) or for allele A of FOXP3 C/A rs3761548 [OR= 1.01 95% CI (0.64-1.58)] (p=0.9797). Meanwhile, for *FOXP3* allelic variants (G + A) in a combined analysis [OR= 6.18; 95% CI (1.67-22.93)] we found a significant association (p=0.0021). (Tables 2 and 3).

Table 2. Case control study association for genotypes of *FOXP3* and *TGFB1*.

GENE	GENOTYPE	OR*	CI 95%	p value[(a)]
FOXP3 A/G rs2232365	AA	1.00	(Reference)	--
	GG	1.07	(0.64-1.79)	0.8100
FOXP3 C/A rs3761548	CC	1.00	(Reference)	--
	AA	0.56	(0.18-1.78)	0.3195
TGFB1 T/C rs1800470	TT	1.00	(Reference)	
	CC	1.88	(1.19-2.96)*	0.0064*
FOXP3 rs2232365/rs3761548	GG/AA	1.15	(0.19-7.00)	0.8773
FOXP3 rs2232365/rs3761548/*TGFB* rs1800470	GG/AA/CC	0.86	(0.08-9.60)	0.9033

[(a)] All analysis were made by *Chi-square (χ^2) test* with a p≤0.05was considered significant
* Odds Ratio (OR) with Confidence Interval (CI) of 95% with a p≤0.05 was considered significant.

Table 3. Case control study for allelic variants of *FOXP3* and *TGFB1* polymorphisms.

GENE	ALLELE	OR*	CI 95%	p value[(a)]
FOXP3 A/G rs2232365	Allele A	1.00	(Reference)	--
	Allele G	1.05	(0.60-1.83)	0.8674
FOXP3 C/A rs3761548	Allele C	1.00	(Reference)	--
	Allele A	1.01	(0.64-1.58)	0.9797
TGFB1 T/C rs1800470	Allele T	1.00	(Reference)	--
	Allele C	4.41	(1.81-10.76)*	0.0005*
FOXP3 rs2232365/rs3761548	Alleles G + A	6.18	(1.67-22.93)*	0.0021*

[(a)] All analysis were made by *Chi-square (χ^2) test* with a p≤0.05was considered significant
* Odds Ratio (OR) with Confidence Interval (CI) of 95% with a p≤0.05 was considered significant.

The analysis of the clinical outcomes of the patients compared to the proposed polymorphisms showed no significant difference, when evaluating *FOXP3* polymorphisms A/G rs2232365 and C/A rs3761548 in relation to clinical pathologic features: hormonal receptor (p=0.250 and p=0.407), tumor size (p=0.789 and p=0.409), lymph node involvement

(p=0.304 and p=0.588), HER-2 (p=0.355 and p=0.199) and clinical staging (p=0.688 and p=0.329), respectively. Also, for *TGFB1* T/C rs1800470 we found no significant results: hormonal receptor (p=0.136), tumor size (p=0.635), lymph node involvement (p=0.828), HER-2 (p=0.988) and clinical staging (p=0.753).

Discussion

Clinic pathological parameters have been validated and serve as a guide for the use of systemic therapy and prognostic. These include tumor size, lymph node stage, histological grade, histological type and the patient's age (LACROIX *et al.*, 2004), molecular profile and response to therapy (RAKHA *et al.*, 2009). Additionally, the majority of samples, both patients and controls, were predominantly Caucasian, a prevalent population in southern Brazil due to European colonization.

Estrogen is a growth factor that stimulates cell proliferation and its effects are mediated through estrogen receptors (ER) (ITO *et al.*, 2010). Approximately 70% of breast cancers are known to express estrogen receptor (ER) and are considered to be hormone-dependent (CIOCCA; ELLEDGE, 2000). HARRIS; SOLIN (2000) observed a range of 47-79% of CDI and 2-15% of invasive lobular carcinoma (CLI) in another breast cancer sample. Since tumor size is directly related to risk of recurrence, we also evaluated this parameter and found that 50% of patients had tumors until 2.0 cm. Tumors of smaller size are invariably associated with a better prognosis for both overall survival and for disease-free survival (ABREU; KOIFMAN, 2002) and the larger its size, the greater the chances of the metastatic involvement of regional lymph nodes (FARLEY; FLANNERY, 1989).

Biological functions of *FOXP3* in tumor cells and its significance remain unclear. Recent data suggest that its expression in tumor cells could be a poor prognostic factor in breast cancer (MERLO *et al.*, 2009). 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 important breast cancer oncogenes and also of CDKN1A (p21) (ZUO, LIU, *et al.*, 2007; ZUO, WANG, *et al.*, 2007; KATOH *et al.*, 2010).

It is known that polymorphisms in *FOXP3* may change this gene functionally, thus leading to the lack of functional Tregs cells, subsequently activated CD4+ T cells which express increased levels of several activation markers and cytokines (WILDIN *et al.*, 2002; BJORNVOLD *et al.*, 2006). Polymorphisms in the promoter region may potentially alter gene expression by changing the binding specificity of transcription factors and by modifying the kinetics of transcription initiation (HANEL *et al.*, 2011).

In this study, two genetic polymorphisms in *FOXP3* promoter region were assessed, but no significant associations for allele carriers or genotypes were found. Our results are in accordance with RASKIN *et al.* (2009), which analyzed three *FOXP3* polymorphisms (rs3761548; rs2294020; rs5906761) in breast cancer patients and also found no association. On the other hand, when analyzed the haplotype for *FOXP3* allele carrier (Allele G + Allele A), we observed a positive association with more than 6 fold in relation to breast cancer susceptibility. At the moment, our study seems to be the first one that made this kind of analysis and we suggest that this gene can act as susceptibility marker in breast tumorigenesis, which must be confirmed in larger samples.

Both tumor and free stromal cells are able to produce cytokines that seem to affect the complex phenomena occurring at the tumor and host interface, thus leading to tumor invasion (COUSSENS; WERB, 2002). Several studies have been conducted suggesting a role for cytokine polymorphisms and other immune molecules in modulating susceptibility to various cancers, including melanoma, prostate, breast, stomach, esophageal and breast cancers (HOWELL *et al.*, 2001; HOWELL *et al.*, 2002; MCCARRON *et al.*, 2002; GIORDANI *et al.*, 2003; SAVAGE *et al.*, 2004).

Transforming growth factor-B (TGFB) is a multifunctional cytokine that regulates cellular processes such as cell division, differentiation, motility, adhesion, and death (KRETZSCHMAR, 2000). In normal cells, TGFB acts as a tumor suppressor by inhibiting cellular proliferation or by promoting cellular differentiation and apoptosis. As cancer develops, them become resistant to the growth-inhibitive properties of TGFB and often increase their production of this cytokine (BLOBE *et al.*, 2000). Given the important roles of TGFB1 in regulating cellular processes, it is biologically plausible that the T/C rs1800470 functional polymorphism may modulate the risk of breast cancer to be associated with increased secretion of this cytokine, which favor cell invasion by suppressing the immune response, angiogenesis, remodeling of extracellular matrix and loss of control of proliferation.

In this work, this polymorphism was assessed and the CC genotype showed significant difference [OR= 1.88 95% CI (1.19-2.96)], as well as C carriers [OR= 4.41 95% CI (1.81-10.76)]. However, the effect of C variant of *TGFB1* polymorphism is still unclear. GU *et al.* (2010) conducted a meta-analysis study of human breast cancer and no significant associations were found for CC vs TT genotype [OR= 1.00, 95% CI (0.92–1.09)], TC vs TT genotype [OR= 0.98, 95% CI (0.93–1.05)], CC/TC vs TT genotypes [OR= 0.99, 95% CI (0.93–1.05)], and CC vs TC/TT genotypes [OR= 1.00, 95% CI (0.93–1.08)]. LE MARCHAND *et al.* (2004) performed an analysis stratified by extent of disease at diagnosis

and found no differences across stages for localized breast cancer [OR= 1.01, 95% CI (0.79–1.29)] and for tumors with regional or distant spread [OR=0.84, 95% CI (0.59–1.19)]. TAUBENSCHUSS *et al.* (2013) also did not find evidence for an association of the same SNP with breast cancer risk [OR= 0.91; 95% CI (0.71–1.16)].

Therefore, JOSHI *et al.* (2011) showed a strong protective effect of C allele in younger western Indian women [OR = 0.45, 95% CI (0.25–0.81)]. DUNNING *et al.* (2003) demonstrated that C allele was associated with higher TGFB1 secretion *in vitro* and corroborating these data, LEE *et al.* (2005) showed that this allele had an increased risk of breast cancer. Another study also showed C allele increasing the risk of breast cancer [OR = 1.3, 95% CI (1.02–1.79)], especially in postmenopausal women [OR= 1.6, 95% CI (1.01–2.44)]. The authors, CHEN *et al.* (2011), proposed that T/C TGFB1 rs1982073 SNP (Single Nucleotide Polymorphism) may contribute to the identification of a more aggressive phenotype among women in North China, since C allele carriers were more likely to bear tumors with histological grade III. Similarly, our results indicated a positive association for CC genotype and for C allele carriers, suggesting this genetic variant as a possible marker of susceptibility in breast cancer development. This may be due to the fact that T/C rs1800470 is located in the signal sequence of TGFB1, and has been suggested to affect the efficiency of its secretion (DUNNING *et al.*, 2003).

MARUYAMA *et al.* (2011) reviewed the progress in understanding the complex molecular events that drive *FOXP3* gene expression and allow functional regulatory T cells to develop. They have discussed the role of transcription factor networks in the expression of *FOXP3* and generation of regulatory T cells and how TGFB is important to initiated *FOXP3* gene expression in naïve T cells. Thus, taking into account the close relationship between the markers discussed in this study, a positive association for *TGFB1* CC genotype (p=0.0064) and for C allele carriers (p=0.0005), as well as for *FOXP3* allelic variants (G + A) in a combined analysis (p=0.0021) was find in relation to breast cancer susceptibility.

Conclusion

TGFB1 showed to be a candidate marker for breast cancer susceptibility, both for the variant allele carrier, as for homozygous mutant genotype. Despite genetic variants of *FOXP3* did not show any significant association with mammary tumorigenesis, the haplotype for the proposed polymorphisms indicated a significant result, which must be interpreted with caution, regarding the size of our sample, but it may present an interesting biological relevance.

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Conflict of interest

The authors declare no conflicts of interests.

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ARTIGO 4

Genetic polymorphisms and expression of *FOXP3* and *TGFB1*: involvement in breast cancer pathogenesis

Delineamento Experimental II

Tecido Tumoral

52 Tecidos

Tecido adjacente normal

Obtenção de células

Extração RNA

Extração DNA

qRT-PCR

Polimorfismos genéticos

RESEARCH ARTICLE

Genetic polymorphisms and expression of *FOXP3* and *TGFB1*: possible involvement in breast cancer pathogenesis

Abstract

The breast tumor microenvironment contains immune cells, cancer cells and their surrounding stroma. The transcription factor forkhead box P3 (FOXP3) seems to be expressed in both tumor and immune cells, especially in regulatory T cells (Tregs), and its expression level is associated with poor patient survival. Transforming growth factor B1 (TGFB1) is an important molecule for generation and function of Tregs in addition to influencing the progression of the tumor at late stages of the disease. Therefore, the aim of this study was to investigate the influence of genetic polymorphisms of *FOXP3* (A/G rs2232365 and C/A rs3761548) and *TGFB1* (T/C rs1800470) and their expression in relation to breast cancer susceptibility and clinical outcome. Genomic DNA and RNA were obtained from 52 tissue of invasive breast carcinoma. There was no association between *FOXP3* and *TGFB1* genetic polymorphisms with clinical outcome. However, there was a relative expression of 15.06 fold higher of FOXP3 in tumor tissue compared to a commercial pool of normal mammary gland RNA. When the relative expression of FOXP3 was compared with polymorphic allelic variants, as well as with clinicopathological parameters, no significant differences were observed. Since the overexpression of TGFB by tumor microenvironment could induce the progression for metastases and, *FOXP3* is involved in the prognostic and survival of patients with breast cancer, our study suggest that the expression of *FOXP3* could be considered a biological marker in breast cancer pathogenesis.

Key words: Breast cancer, *FOXP3*, *TGFB1*, gene expression.

Introduction

Breast cancer is the leading cause of cancer death among women worldwide and data from the National Cancer Institute (BRAZIL, 2013) reveal that in Brazil are expected 57,120 new cases per year. It is worth noting that, regardless the type of non-melanoma cancer, the mammary tumor is the most common among women in the Southeast, South, Midwest and Northeast regions of Brazil, accounting for a high rate of morbidity and mortality. This neoplasia is a heterogeneous disease with different known biological subclasses (CALLAGY *et al.*, 2003), some of which are associated with immune cell infiltration (HRUBISKO *et al.*, 2010).

The tumor microenvironment contains innate immune cells (including macrophages, neutrophils, mast cells, myeloid derived suppressor cells, dendritic cells, and natural killer cells) and adaptative immune cells (T and B lymphocytes) in addition to the cancer cells and their surrounding stroma (which consists of fibroblasts, endothelial cells, pericytes, and mesenchymal cells) (DE VISSER *et al.*, 2006). The presence of leukocytes within tumors provided the first indication of a possible link between inflammation and cancer (KARIN, 2006). Human regulatory T cells, characterized as CD4+CD25+ T cells with suppressive function, are found in peripheral blood (BAECHER-ALLAN *et al.*, 2001; DIECKMANN *et al.*, 2001; JONULEIT *et al.*, 2001) and in the microenvironment of tumor (KNUTSON *et al.*, 2007).

A transcription factor, called FOXP3, was demonstrated to be expressed by these cells (HORI *et al.*, 2003; FONTENOT *et al.*, 2005). Polymorphisms have been described in various regions of the *FOXP3* gene, such as the promoter, intron and exon regions (ODA *et al.*, 2013). Critical for the initiation and regulation of FOXP3 transcription is a conserved promoter sequence, located upstream of the transcriptional start site (SAKAGUCHI *et al.*, 2008), that contains two polymorphisms, C/A rs3761548 and A/G rs2232365 (GAO *et al.*, 2010; ZHANG *et al.*, 2012; JAHAN, CHERUVU, *et al.*, 2013; YANG *et al.*, 2013; ZHANG *et al.*, 2013). Several studies in primary breast tumors have shown that a high number of tumor infiltrating Treg cells within lymphoid infiltrates surrounding the tumor was predictive of an early relapse and death (GOBERT *et al.*, 2009; MERLO *et al.*, 2009; OHARA *et al.*, 2009). Moreover, MERLO *et al.* (2009) suggest that FOXP3 is also expressed in breast cancer cells, and the expression level is associated with patient survival.

It is known that cytokines are small molecules secreted by cells in response to specific stimuli and alter the behavior of the same or other cells (HOWELL; ROSE-ZERILLI, 2007). In addition, transforming growth factor beta (TGFB) plays an important role in the generation

of regulatory T cells (Tregs) from CD4+CD25- precursors (FU *et al.*, 2004). The TGFB family of polypeptides comprises a group of highly conserved dimeric proteins with a molecular weight of approximately 25 kDa (ROBERTS; SPORN, 1993). Genes encoding components of the TGFB signaling pathway, including TGFB1 (PASCHE *et al.*, 1999), TGFBRI (BEISNER *et al.*, 2006) and TGFB2 (CAMBIEN *et al.*, 1996), are functionally polymorphic in humans. TGFB1 harbors promoter and signal peptide polymorphisms that influence protein secretion and levels of freely circulating TGFB1 (PASCHE *et al.*, 1999; DUNNING *et al.*, 2003). Several groups have demonstrated an association between variant *TGFB*1 alleles and breast cancer risk (PASCHE *et al.*, 1999; ZIV *et al.*, 2001; HISHIDA *et al.*, 2003).

Among them, a T to C transition at nucleotide 29 of amino acid number 10 changes leucine to proline and is termed T+29C (Leu10Pro, 869T>C, T869C, T/C rs1800470). This transition disrupts the structure (CAMBIEN *et al.*, 1996) and results in increased levels of TGFB protein (SUTHANTHIRAN *et al.*, 2000; DUNNING *et al.*, 2003) and mRNA (SUTHANTHIRAN *et al.*, 2000)

For this reason, in the current study, our aim was to evaluate polymorphisms in *FOXP3* (A/G rs2232365, A/C rs3761548) and *TGFB* (T/C rs1800470) and their respective gene expression in tumoral and normal breast tissue, in the search for a correlation with breast cancer pathogenesis.

Materials and Methods

Human Subjects and samples

Following approval from the Human Ethics Committee of the State University of Londrina (CEP/UEL No 233/09), which is in accordance with the National Committee for Ethics in Research (CONEP-CAAE - 0179.0.268-09) and Resolution 196/96 - National Health Council (CNS), the patients were invited to participate, informed in detail regarding the research and a voluntary written consent term was obtained. Clinical staging was determined according to the Union of International Control of Cancer (UICC, 2002) classification criteria.

Samples of invasive breast carcinoma tissue were obtained from a case series of 52 patients who had undergone surgery at the Cancer Hospital of Londrina, Parana State, Brazil. All tissue samples obtained were free of adjuvant or neoadjuvant chemotherapy. The tumor-node-metastasis (TNM) system was used to classify cancer based on the major morphological attributes of malignant tumors that were thought to influence disease prognosis: size of the

primary tumor (T), presence and extent of regional lymph node involvement (N) and presence of distant metastases (M).

DNA extraction

Genomic DNA was obtained from tissue samples of invasive breast carcinoma by salting-out method (KIRBY, 1993) and were quantitated by Thermo Fisher Scientific NanoDrop 2000c®Spectrophotometer (USA) at a wavelength of 260nm and 280nm.

Genetic Polymorphisms

DNA (100 ng) was amplified by allelic specific polymerase chain reactions (AS-PCR) with specific primers for *FOXP3* gene (A/G, rs2232365; C/A, rs3761548) according to GAO *et al.* (2010) modified, which the cycles condition where reduced and for *TGFB* (T/C rs1800470), according to LEE *et al.* (2005) modified, which reagents ($MgCl_2$) conditions were increased to allele T (Table 1), following the GenBank accession numbers NT_079573.4 and NG_013364.1, respectively. All the reactions were performed with positive and negative controls.

Table 1. Allelic variants and amplicons of *FOXP3* and *TGFB1* genes.

GENE	ALLELE	PCR PRODUCT
FOXP3		
-924 (A/G, rs2232365)	(A)	442bp
	(G)	427bp
-3279 (C/A, rs3761548)	(C)	333bp
	(A)	334bp
TGFB		
+869 (T/C, rs1800470)	(T)	297bp
	(C)	204bp

RNA isolation and reverse transcriptase reaction

Total cellular RNA was obtained from 52 samples of breast tumor tissue, using TRIzol LS reagent (InvitrogenTM, Carlsbad, California, USA) according to manufacturer's instructions. A commercial pool of normal mammary gland RNA (Clontech, Human mammary gland, total RNA, Takara Biotechnology (Dalian), CO., LTD.) was used to perform the relative expression. Purified total RNA was measured and assessed for purity by determining absorbance at 260 and 280nm and were quantitated by Thermo Fisher Scientific NanoDrop 2000c®Spectrophotometer (USA). Reverse transcriptase reaction was performed

using 500ng of RNA, 20 units of cloned Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT; Invitrogen™), 4 units of Recombinant Ribonuclease Inhibitor (RNaseOUT™; Invitrogen™) under the following conditions: 2.5 μM oligo dT, 50mM Tris HCl pH 8.3, 75 mM KCl, 1.5 mM $MgCl_2$, 1.25 mM of dNTP, at 42^{o}C for 60 min in a Hybaid PCR Sprint Thermal Cycler (Biosystems, Guelph, Ontario, Canada).'

Molecular analysis of beta-actin mRNA

Integrity of RNA samples and cDNA quality were analyzed by conventional PCR for beta-actin with specific primers as described by AMARANTE *et al.* (2005). PCR conditions were: 94ºC for 1 min followed by 35 cycles of 94ºC for 30 sec, 55ºC for 30 sec, 72º C for 1 min and finally, 72ºC for 10 min in a MG96 - Biocycler (imported by Biosystems, Curitiba PR, Brazil). All the RNA samples used presented detectable quantities of beta-actin mRNA and acceptable integrity during amplification. No contamination with genomic DNA was verified, since all the amplified products presented a fragment correspondent to 353bp.

Real-Time PCR (qPCR) for FOXP3 mRNA and TGFB mRNA

Quantitative Real Time-PCR (RT-PCR) was performed using Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen) and Step One™ Real-Time PCR Systems (Applied Biosystems, Foster City, USA) (Table 2). Real-time PCR using SYBR green fluorescence was performed with 20ng of cDNA in a total volume of 20μL. The thermal cycling conditions were 10 minutes at 95°C, and 40 cycles of 15 seconds at 95°C and 1 minute at 60°C, followed by melting curve, which consisted of one cycle of 15 seconds at 95°C, 1 minute at 60°C and growing increase up to 95°C (0,3°C/second) with detection of fluorescence at each temperature increase to confirm the specific amplification.

Table 2. Quantitative RT-PCR primers sequences for target (*FOXP3* and *TGFB*) and constitutive (18S) genes.

Gene	GenBank AN*	Primer	Sequence
FOXP3 mRNA	NM_014009	*Foward*	5′ CTGCCCCTAGTCATGGTGG 3’
		Reverse	5′ CTGGAGGAGTGCCTGTAAGTG 3′
TGFB mRNA	NM_ 000660	*Foward*	5’ GTC GGG AGA AGA GGA AAA AAA 3’
		Reverse	5’ GGC AAA GGG AGG CGG TC 3’
18S mRNA	NR_003286	*Foward*	5’ GTAACCCGTTGAACCCCATT 3’
		Reverse	5’ CCATCCAATCGGTAGTAGCG 3’

GenBank AN = accession number*

In quantitative RT-PCR analysis, the expression levels of *FOXP3* and *TGFB* mRNA were calculated according to Delta Delta Ct (ΔΔCt) method, in which Ct values for target genes were the mean fold change + SEM for three independent determinations, corrected by ribosomal RNA (18S) Ct values from control samples. The relative expression was calculated by $2^{-\Delta CT}$ method to determine the effect of internal control gene (18S); the relative expression levels of genes *FOXP3* and *TGFB1* were calculated using the $2^{-\Delta\Delta CT}$ method (LIVAK; SCHMITTGEN, 2001).

Statistical Analysis

Statistical analysis was realized using Kruskal-Wallis test to compare polymorphisms and clinic pathological parameters and χ^2 test to Hardy-Weinberg Equilibrium in SPSS Statistics 17.0 software (SPSS inc., Chicago, Illinois, USA) and the GraphPad Prism 5.0 Software. A p value ≤ 0.05 was considered statistically significant.

Results

Clinic pathological features

The age of patients involved in the present study ranged from 38 to 81 years, and the highest frequency of breast cancer was observed in women aged over 64 years. Most patients showed invasive ductal carcinoma (94.23%, 49/52). Tumor size ranged from 0.1-6.8 cm. The positivity of hormonal receptors was observed in 90.38% (47/52) of the patients (Table 3).

Table 3. Distribution of clinical and pathological characteristics of 52 breast cancer tissue.

Clinical and Pathological Features		Sample	
		n	(%)
Age (years) (n=41)	< 40 years	1	2.4
	41 - 50 years	15	36.6
	51 - 60 years	6	14.6
	> 60 years	19	46.3
Tumor Size (n=52)	0 - 2.0 cm	31	58.5
	2.1 - 5.0 cm	16	30.2
	> 5.0 cm	5	9.4
Lymph node metastasis (n=50)	Positive	19	38.0
	Negative	31	62.0
RH (n=36)	Positive	32	88.9
	Negative	4	11.1
p53 (n=36)	Positive	11	30.6
	Negative	25	69.4
HER2 (n=33)	Positive	7	21.2
	Negative	26	78.8
Ki67 (n=36)	Low	18	50.0
	Moderate	5	13.9
	High	13	36.1

TGFB and FOXP3 polymorphism analysis

The genotypes distributions of *FOXP3* (A/G rs2232365 and C/A rs3761548) and *TGFB*1 (T/C rs1800470) genetic variants in tumors specimens are shown in Table 4.

Table 4. Genotype distribution of *TGFB*1 and FOXP3 polymorphisms in 52 breast cancer tissues samples.

Gene, SNP description, and rs	Genotype	Patients N (%)	χ^2 value*
FOXP3, -924, A/G rs2232365	AA	16 (30.77)	0.6573
	AG	23 (44.23)	
	GG	13 (25.00)	
FOXP3, -3279, C/A rs3761548	CC	25 (48.08)	0.00250
	CA	22 (42.31)	
	AA	5 (9.62)	
*TGFB*1, +869, T/C rs1800470	TT	08 (15.38)	2.1520
	TC	31 (59.62)	
	CC	13 (25.00)	

∗ The analysis of the Hardy-Weinberg Equilibrium for the polymorphisms FOXP3A/G rs2232365, FOXP3C/A rs3761548 and TGFB1 T/C rs18004 showed that all samples are in equilibrium ($p>0.05$).

Clinical and biological characteristics of women with breast cancer were analyzed in relation to presence or absence of genotypic variants: hormonal receptor status, p53 expression, Ki67 index, lymph node involvement, tumor size and nuclear grade. The distribution of genotype polymorphisms according to clinical outcome is presented in Table 5.

We performed a genotypic analysis by Kruskal-Wallis test and there was no association between *FOXP3* A/G rs2232365 and C/A rs3761548 polymorphisms compared to HER-2 ($p=0.520$ and $p=0.421$), p53 ($p=0.670$ and $p=0.698$), Ki67 ($p=0.100$ and $p=0.150$), hormone receptors ($p=0.178$ and $p=0.258$), lymph node metastasis ($p=0.811$ and $p=0.494$), cancer type ($p=0.724$ and $p=0.055$), tumor size ($p=0.408$ and $p=0.345$), age range ($p=0.659$ and $p=0.170$), nuclear grade ($p=0.324$ and $p=0.150$), respectively. Likewise, genotype analysis of *TGFB* T/C rs1800470 polymorphism compared to outcome clinical characteristics did not show significant difference: HER-2 ($p=0.392$), p53 ($p=0.353$), Ki67 ($p=0.380$), hormone receptors ($p=0.445$), lymph node metastasis ($p=0.400$), cancer type ($p=0.504$), tumor size ($p=0.298$), age range ($p=0.068$) and nuclear grade ($p=0.567$).

Table 5. Genotypic distribution in relation to clinical outcome and molecular characteristics of breast cancer tissue.

		***FOXP3* A/G (rs2232365)**				***FOXP3* C/A (rs3761548)**				***TGFB* T/C (rs1800470)**			
		AA	**AG**	**GG**		**CC**	**CA**	**AA**		**TT**	**TC**	**CC**	
		N (%)	**N (%)**	**N (%)**	**p value[#]**	**N (%)**	**N (%)**	**N (%)**	**p value[#]**	**N (%)**	**N (%)**	**N (%)**	**p value[#]**
Type Breast Cancer (n=52)	IDC*	7 (13.2)	31 (58.5)	11 (20.8)		19 (35.8)	29 (54.7)	1 (1.9)		7 (13.7)	28 (54.9)	13 (25.5)	
	ILC**	0 (0.0)	2 (3.8)	0 (0.0)	0.724	1 (1.9)	1 (1.9)	0 (0.0)	0.055	1 (2.0)	0 (0.0)	0 (0.0)	0.504
	Special***	0 (0.0)	1 (1.9)	1 (1.9)		0 (0.0)	1 (1.9)	1 (1.9)		0 (0.0)	2 (3.9)	0 (0.0)	
Tumor Size (n=52)	0.1-2.0 cm	4 (7.7)	22 (42.3)	5 (9.6)		10 (19.2)	19 (36.5)	2 (3.8)		4 (8.0)	15 (30.0)	10 (20.0)	
	2.1-4.0 cm	1 (1.9)	8 (15.4)	6 (11.5)		7 (13.5)	8 (15.4)	0 (0.0)		2 (4.0)	11 (22.0)	2 (4.0)	
	4.1-6.0 cm	2 (3.8)	2 (3.8)	1 (1.9)	0.408	3 (5.8)	2 (3.8)	0 (0.0)	0.345	2 (4.0)	2 (4.0)	1 (2.0)	0.298
	> 6.1 cm	0 (0.0)	1 (1.9)	0 (0.0)		0 (0.0)	1 (1.9)	0 (0.0)		0 (0.0)	1 (2.0)	0 (0.0)	
Age Range (n=49)	< 40 years	0 (0.0)	1 (2.0)	0 (0.0)		0 (0.0)	1 (2.0)	0 (0.0)		0 (0.0)	0 (0.0)	1 (2.0)	
	41-50 years	2 (3.9)	12 (23.5)	3 (5.9)		4 (7.8)	12 (23.5)	1 (2.0)		1 (2.0)	11 (22.4)	5 (10.2)	
	51-60 years	1 (2.0)	5 (9.8)	3 (5.9)	0.659	3 (5.9)	6 (911.8)	0 (0.0)	0.170	0 (0.0)	5 (10.2)	2 (4.1)	0.068
	> 60 years	4 (7.8)	14 (27.5)	6 (11.8)		12 (23.5)	11 (21.6)	1 (2.0)		7 (14.3)	13 (26.5)	4 (8.2)	
Nuclear Grade (n=52)	I	1 (2.1)	7 (14.6)	3 (6.3)		5 (10.4)	6 (12.5)	0 (0.0)		1 (2.1)	5 (10.6)	4 (8.5)	
	II	2 (4.2)	10 (20.8)	6 912.5)	0.324	10 (20.8)	8 (16.7)	0 (0.0)	0.150	2 (4.3)	11 (23.4)	5 (10.6)	0.567
	III	4 (8.3)	13 (27.1)	2 (4.2)		4 (8.3)	14 (29.2)	1 (2.1)		3 (6.4)	12 (25.5)	4 (8.5)	
RH (n=52)	Positive	3 (5.8)	32 (61.5)	10 (19.2)		16 (30.8)	28 (53.8)	1 (1.9)		5 (10.0)	27 (54.0)	11 (22.0)	
	Negative	4 (7.7)	2 (3.8)	1 (1.9)	0.178	3 (5.8)	3 (5.8)	1 (1.9)	0.258	2 (4.0)	3 (6.0)	2 (4.0)	0.445
HER2 (n=49)	Positive	3 (6.1)	10 (20.4)	0 (0.0)		3 (6.1)	9 (18.4)	1 (2.0)		1 (2.1)	7 (14.9)	5 (10.6)	
	Negative	3 (6.1)	22 (44.9)	11 (22.4)	0.520	15 (30.6)	20 (40.8)	1 (2.0)	0.421	6 (12.8)	21 (44.7)	7 (14.9)	0.392
p53 (n=47)	Positive	3 (6.4)	9 (19.1)	0 (0.0)		4 (8.5)	7 (14.9)	1 (2.1)		2 (4.4)	5 (11.1)	5 (11.1)	
	Negative	3 (6.4)	22 (46.8)	10 (21.3)	0.670	14 (29.8)	20 (42.6)	1 (2.1)	0.698	5 (11.1)	21 (46.7)	7 (15.6)	0.353
Ki67 (n=39)	Low	1 (2.4)	10 (24.4)	7 (17.1)		10 (24.4)	7 (17.1)	1 (2.4)		4 (10.3)	9 (23.1)	4 (10.3)	
	Moderate	1 (2.4)	4 (9.8)	1 (2.4)	0.100	2 (4.9)	4 (9.8)	0 (0.0)	0.150	2 (5.1)	3 (7.7)	0 (0.0)	0.380
	High	4 (9.8)	11 (26.8)	2 (4.9)		4 (9.8)	12 (29.3)	1 (2.4)		1 (2.6)	10 (25.6)	6 (15.4)	
Lymph node metastasis (n=48)	Positive	2 (4.2)	13 (27.1)	4 (8.3)	0.811	7 (14.6)	12 (25.0)	0 (0.0)	0.494	2 (4.3)	13 (28.3)	4 (8.7)	0.400
	Negative	5 (10.4)	18 (37.5)	6 (12.5)		11 (22.9)	16 (33.3)	2 (4.2)		5 (10.9)	13 (28.3)	9 (19.6)	

*IDC = Invasive Ductal Carcinoma; ** ILC = Invasive Lobular Carcinoma; ***Special = Adenocarcinoma

[#]Kruskal-Wallis test.

***Relative expression of TGFB and FOXP3* genes**

The relative expression of *FOXP3* and *TGFB1* was performed with a commercial pool of normal mammary gland RNA Clontech, Human mammary gland, total RNA, Takara Biotechnology (Dalian), CO., LTD.). For *TGFB1*, breast tumor tissues showed an expression of this gene, however, the commercial pool of normal mammary gland did not show any *TGFB1* expression, not allowing the calculation. For *FOXP3* gene an increase of 15.06 (±3.44) (average ± standard deviation) fold was observed by average and 6.161 (±3.621) (median ± 25 percentile) fold by median, as shown in Figure 1.

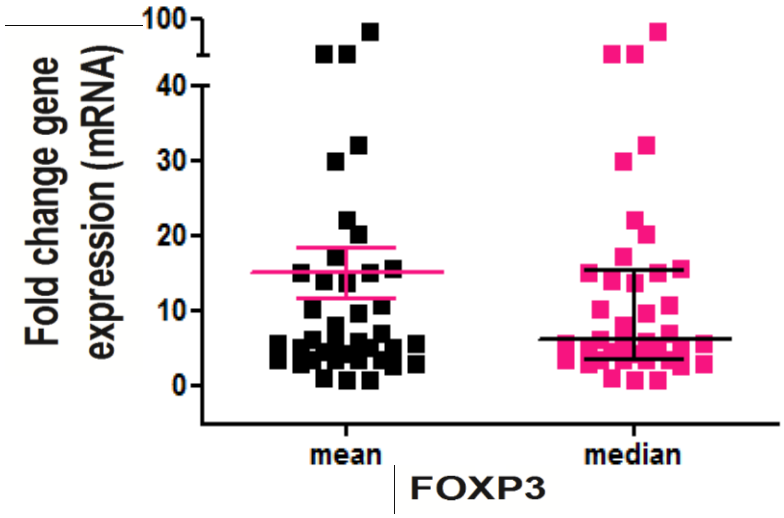

Figure 1. Relative expression of *FOXP3* gene in breast tissue. The expression levels of *FOXP3* mRNA were calculated according to DeltaDeltaCt (ΔΔCt) method, in which Ct values for target genes were the mean fold change + SEM for three independent determinations corrected by ribosomal RNA (18S). The commercial pool of normal mammary gland RNA was used as control (Clontech, Human mammary gland, total RNA, Takara Biotechnology (Dalian), CO., LTD.)

There was no significant association between *FOXP3* mRNA expression in relation to hormonal receptor (p=0.403), HER-2 expression (p=0.215), p53 expression (p=0.142), lymph node metastasis (p=0.605), Ki67 expression (p=0.128), tumor size (p=0.833), age range (p=0.396) or with genotypic variants of *FOXP3* (A/G rs2232365 and C/A rs3761548) and *TGFB* (T/C rs1800470) with p=0.969, p=0.913 and p=0.898, by Kruskal-Wallis test, respectively (Figure 2).

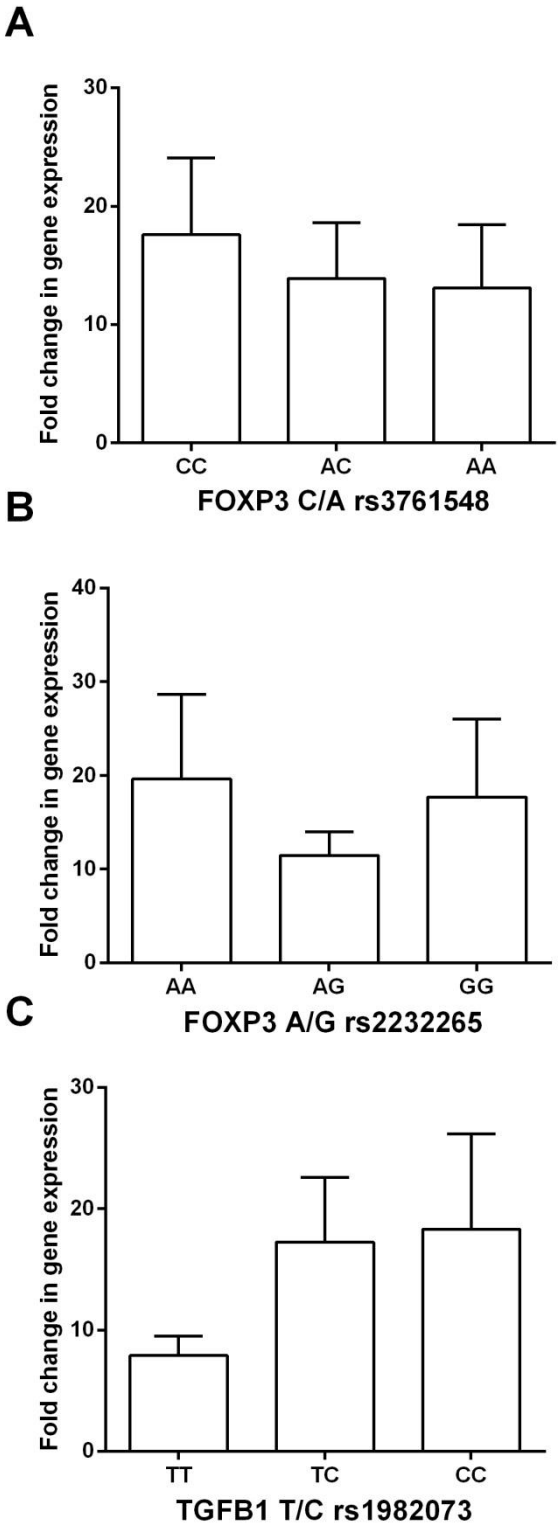

Figure 2. Relative expression of *FOXP3* gene in relation to genetic polymorphisms: (A) C/A rs3761548; (B) A/G rs2232365 and (C) TGFB1 T/C rs1800470. The evaluation of relative expression of *FOXP3* with genotypes was performed by Kruskal-Wallis test and no significant difference was observed. The relative expression of *FOXP3* with genetic polymorphisms showed a p=0.969, p=0.913 and p=0.898 to A/G rs2232365; C/A rs3761548 and TGFB1 T/C rs1800470, respectively.

Discussion

The proliferation of malignant breast epithelial cells is regulated by various stimuli including cytokines and growth factors, thus the variants of these genes may modify the individual breast cancer risk (LEE *et al.*, 2005). In the present study, there was no association between *FOXP3* (A/G rs2232365 and C/A rs3761548) and *TGFB1* (T/C rs1800470) polymorphisms with parameters of clinical outcome. Previous results of our group are in accordance with these data, since ODA *et al.* (2012) did not find any significant differences between *TGFB*1 genotype distribution and clinic pathological characteristics.

FOXP3 is a member of the forkhead family of transcription factors critically involved in the development and functions of Treg cells, and is, thus far, the most specific marker of this cell subtype (COFFER; BURGERING, 2004). Until recently, FOXP3 expression was thought to occur mainly in these cells subtypes and some populations of activated T cells. However, recent studies have provided clear evidence that various types of human cancer cells, including breast cancer, expressed the transcript of FOXP3, as well as the mature protein (HINZ *et al.*, 2007; KARANIKAS *et al.*, 2008; LADOIRE *et al.*, 2011).

According to OHARA *et al.* (2009) the *FOXP3* mRNA expression in cancer tissue was significantly up regulated when compared with normal breast tissue expression ($p< 0.01$), even at the ductal carcinoma *in situ* (DCIS) stage ($P < 0.01$). Corroborating this data, we observed a relative expression of 15.06 fold higher of *FOXP3* mRNA in tumor tissue compared with a commercial pool of normal mammary gland RNA. Our results are in accordance with GUPTA *et al.* (2007) that showed a higher relative expression of *FOXP3* gene in infiltrating breast carcinoma (7.43±3.44) compared with ductal carcinoma *in situ* (4.27±1.97) and in normal tissues (3.51±1.22) ($p<0.001$). Therefore, the *FOXP3* expression appears to be associated with the invasive phenotype of human breast carcinoma as compared with the non-invasive phenotype.

Studies have shown that Treg cells may mediate immunosuppression through the secretion of TGFB (CHEN *et al.*, 2005). However, this cytokine is also produced from not only Treg cells, but many cell types including tumor cells, and it is implicated in several aspects of breast cancer onset and progression (WAKEFIELD *et al.*, 2001). OHARA *et al.* (2009) showed that *TGFB1* expression in cancer tissue were significantly up regulated when compared with normal breast tissue ($p<0.001$), even at the ductal carcinoma *in situ* (DCIS) stage ($p<0.001$). Similarly, HARTMANN *et al.* (2011) showed *TGFB*1 gene expression was significantly higher in breast tumor tissue compared to normal tissue ($p < 0.0001$). Unfortunately, the analysis of the relative expression of *TGFB1* in this work cannot be

performed by the fact that normal tissue used for comparison had not shown detectable expression. Therefore, since *TGFB1* have been expressed in tumor tissue and not in normal tissue, it is suggested that this gene may have its expression differently regulated in the tumor microenvironment.

We also analyzed the *FOXP3* expression in relation to clinical pathological parameters, including tumor size, lymph node involvement, HER-2 and hormonal receptor status and we did not find any significant association. Likewise, GUPTA *et al.* (2007) also did not find correlation between the *FOXP3* gene expression and the clinic pathological features, including lymph node metastasis. On the other hand, OHARA *et al.* (2009) did not find correlation between *FOXP3* expression and lymphatic invasion score, however showed that *FOXP3* mRNA expression was positively correlated with tumor grade and also significantly higher in PR-negative cancers or HER2-positive cancers.

Conclusion

Our study demonstrated an overexpression of *FOXP3* gene in tumor compared with normal tissue, suggesting that this gene can be differently regulated in tumor microenvironment and may have implication in breast tumorigenesis.

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Conflict of interest

The authors declare no conflicts of interests.

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CONCLUSÃO

Artigo 1

- Sabe-se que o fator de crescimento de transformação beta (TGFB) pode atuar tanto como supressor tumoral, nos estágios iniciais da doença, e como um estimulador importante da progressão do tumor, invasão e metástase, em fases avançadas. As Tregs podem ser divididas em dois subgrupos principais: Tregs natural (nTreg) e Tregs adaptativas ou induzidas (iTreg). O perfil de citocinas do microambiente tumoral tem profunda influência sobre a manutenção ou a progressão dos tumores malignos, pois pode atrair nTreg do tecido periférico e induzir células T CD4+ do próprio tumor primário a se diferenciar em iTreg, favorecendo o processo de tumorigênese (invasão celular e metástase).

Artigo 2

- O gene *FOXP3* é uma importante molécula para a função das Tregs, tanto no contexto da manutenção da tolerância quanto na regulação da resposta imunológica. Polimorfismos nesse gene podem levar ao desequilíbrio do sistema imune e mediar o desenvolvimento de doenças humanas graves. O estudo destes polimorfismos pode contribuir para uma melhor compreensão da patogênese e progressão das doenças, principalmente nas autoimunes e nos cânceres.

Artigo 3

- O estudo de associação caso-controle indicou uma associação positiva para o polimorfismo analisado do gene *TGFB1*, tanto para o homozigoto CC quanto para o portador do alelo C.
- Nenhuma associação significativa foi encontrada entre os polimorfismos de *FOXP3* (A/G rs2232365, C/A rs3761548) quando estes foram analisados de forma isolada.
- Entretanto, quando as variantes alélicas de *FOXP3* foram analisadas de forma combinada, foi observada uma associação positiva em relação á suscetibilidade ao câncer de mama.
- Não foi observada diferença significativa quando foram avaliados os polimorfismos de *FOXP3* e *TGFB1* com as características clínicopatológicas.

Artigo 4

- Não houve associação entre os polimorfismos genéticos de *FOXP3* e *TGFB1* em relação aos parâmetros clínicopatológicos das pacientes.
- Foi observada uma expressão relativa de 15,06 vezes superior do RNAm de *FOXP3* no tecido tumoral em comparação com o *pool* comercial de glândula mamária normal considerando a média ± desvio padrão e uma expressão relativa de 6.161 vezes superior considerando a mediana ± percentil de 25%.
- A análise da expressão relativa de *FOXP3* não mostrou diferença significativa em relação às variantes alélicas polimórficas, e nem em relação às características clínicopatológicas das pacientes.

CONSIDERAÇÃO FINAL

Segundo os dados da literatura, resumidos em nossas revisões, a superexpressão de TGFB no microambiente tumoral pode induzir metástases e o gene *FOXP3* está envolvido na manutenção da tolerância e na regulação da resposta imune. Assim, um desequilíbrio nesse sistema pode influenciar o prognóstico e a sobrevida das pacientes com câncer de mama. Adicionalmente, nosso estudo sugere que os polimorfismos genéticos e a expressão de *FOXP3* e *TGFB1* podem ser considerados marcadores biológicos de suscetibilidade e progressão do câncer de mama, devendo os mesmos ser estudados em amostras maiores para a confirmação e validação destes resultados.

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ANEXOS

CLASSIFICAÇÃO DO SISTEMA TUMOR-NÓDULO-METÁSTASE (TNM) 7ª EDIÇÃO

Tabela 5. Classificação histológica do Tumor Primário (T)[a,b]

TX	Tumor primário não pode ser avaliado
T0	Não há evidência de tumor primário
Tis	Carcinoma *in situ*.
Tis (DCIS)	Carcinoma Ductal *in situ* (DCIS).
Tis (LCIS)	Carcinoma Lobular *in situ* (LCIS).
Tis (Paget)	Doença de Paget do mamilo não associado com carcinoma invasivo e / ou carcinoma *in situ* (DCIS e/ou LCI) no parênquima mamário subjacente. Os carcinomas do parênquima da mama associados com a doença de Paget são classificados com base na dimensão (tamanho) e nas características da doença do parênquima, embora a presença da doença de Paget ainda possa ser notada.
T1	Tumor ≤20 mm em sua maior dimensão
T1mi	Tumor ≤1 mm em sua maior dimensão
T1a	Tumor >1 mm, mas ≤5 mm em sua maior dimensão
T1b	Tumor >5 mm, mas ≤10 mm em sua maior dimensão
T1c	Tumor >10 mm, mas ≤20 mm em sua maior dimensão
T2	Tumor > 20 mm, mas ≤ 50 mm em sua maior dimensão
T3	Tumor >50 mm em sua maior dimensão
T4	Tumor de qualquer tamanho com extensão direta à parede torácica e /ou para a pele (ulceração ou nódulos da pele)[c]
T4a	Extensão à parede torácica, não incluindo apenas o músculo peitoral adesão / invasão.
T4b	Ulceração e /ou nódulos satélites ipsilaterais e /ou edema (incluindo peau d'orange) da pele, que não preenchem os critérios para o carcinoma inflamatório.
T4c	Ambos T4a e T4b.
T4d	Carcinoma inflamatório

DCIS = carcinoma ductal in situ; LCIS = carcinoma lobular in situ.

[a] *Reproduzido com a permissão do AJCC: mama. In: Borda SB, Byrd DR, Compton CC, et al, eds:..Manual de Estadiamento do Câncer AJCC. 7 ed. New York, NY: Springer, 2010, pp 347-76.*

[b] *A classificação T do tumor primário é o mesmo independentemente do fato de se basear em critérios clínicos ou patológicos, ou ambos. Tamanho deve ser medido em milímetro. Se o tamanho do tumor é ligeiramente inferior ou superior a um ponto de corte para uma dada classificação T, recomenda-se que o tamanho seja arredondado para a leitura mais próxima do ponto de corte. Por exemplo, o tamanho relatado de 1,1 mm, é reportado como um milímetro, ou um tamanho de 2,01 cm é reportado*

como 2,0 centímetros. Designação deve ser feita com o índice "c" ou "p" para indicar se a classificação T foi determinada pelo clínico (exame físico ou radiológico) ou patológicos, respectivamente. Em geral, a determinação patológica deve ter precedência sobre a determinação clínica do tamanho T.
[c] *Somente invasão da derme não se qualifica como T4.*

Tabela 6. Classificação clínica dos linfonodos regionais (N)[a]

NX	Os linfonodos regionais não podem ser avaliados (e.g., removido previamente).
N0	Sem metástase para linfonodo(s) regional(is).
N1	Metástase para linfonodo(s) ipsilateral(is) móvel(is) nível I ou axilar nível II.
N2	Metástase para linfonodo(s) ipsilateral(is) fixo(s) nível I, axilar(es) nível II ou linfonodo(s) mamário(s) interno(s) ipsilateral(is) na ausência de metástase para linfonodo(s) axilar(es) evidente(s).
N2a	Metástase para linfonodo(s) ipsilateral(is) nível I ou axilar(es) nível II, fixo(s) entre si ou fixo(s) a outras estruturas.
N2b	Metástase somente para linfonodo(s) mamário(s) ipsilateral(is) interno(s) e ausência de acometimento de linfonodo(s) axilar(es) nível I e II.
N3	Metástase para linfonodo(s) ipsilateral(is) infraclavicular(ES) (nível III axilar), com ou sem acometimento de linfonodo(s) axilar(es) níveis I e II; ou presença de linfonodo(s) ipsilateral(is) mamário(s) interno(s) com acometimento de linfonodo(s) axilar(es) nível I e II; ou metástase para linfonodo(s) supraclavicular(es) ipsilateral(is) com ou sem linfonodo(s) axilar(es) ou mamário(s) interno(s).
N3a	Metástase para linfonodo(s) ipsilateral(is) infraclavicular(ES) (nível III).
N3b	Metástase para linfonodo(s) mamário(s) ipsilateral(ais) interno(s) e axilar(es).
N3c	Metástase para linfonodo(s) ipsilateral(ais) supraclavicular(es)

[a]*Reproduzido com permissão do AJCC: Breast. In: Edge SB, Byrd DR, Compton CC, et al., eds.: AJCC Cancer Staging Manual. 7th ed. New York, NY: Springer, 2010, pp 347-76.*
[b] *Linfonodos clinicamente detectados são aqueles detectados por meio de exames por imagem (exceto linfocitilografia) ou por meio do exame clínico tendo características altamente suspeitas de malignidade ou uma macrometástase patológica baseada em uma punção aspirativa por agulha fina com exame citológico. Confirmação de doença metastática clinicamente detectada por aspiração por agulha final sem biópsia por excisão é designada com um sufixo (f), por exemplo, cN3a(f). Biópsia excisional de um linfonodo ou biópsia de um linfonodo sentinela, na ausência de atribuição patológica pT, é classificada como N, por exemplo, cN1. Informação relativa a confirmação do status nodal será designado em fatores específicos do local, como clínico, aspiração por agulha fina, biópsia de núcleo ou biópsia de linfonodo sentinela. Classificação patológica (pN) é utilizada para excisão ou biópsia do linfonodo sentinela apenas em altamente suspeitas de malignidade ou uma macrometástase patológica baseada em uma punção aspirativa por agulha fina com exame citológico. Confirmação de doença metastática clinicamente detectada por aspiração por agulha final sem biópsia por excisão é designada com um sufixo (f), por exemplo, cN3a(f). Biópsia excisional de um*

linfonodo ou biópsia de um linfonodo sentinela, na ausência de atribuição patológica pT, é classificada como N, por exemplo, cN1. Informação relativa a confirmação do status nodal será designado em fatores específicos do local, como clínico, aspiração por agulha fina, biópsia de núcleo ou biópsia de linfonodo sentinela. Classificação patológica (pN) é utilizada para excisão ou biópsia do linfonodo sentinela apenas em combinação com uma atribuição patológica T.

Tabela 7. Classificação patológica dos linfonodos regionais (pN)[a,b]

pNX	Linfonodos regionais não podem ser acessados (e.g., previamente removidos ou não ressecados para estudo patológico).
pN0	Ausência de metástase para linfonodo(s) regional(is) identificado(s) histologicamente.
pN0(i–)	Ausência de metástase para linfonodo(s) regional(is) em exame histológico e ausência de aglomerados isolados de células tumorais.
pN0(i+)	Linfonodos regionais com aglomerados de células malignas menores que 0,2mm, detectado por H&E ou IHC incluindo ITC.
pN0(mol–)	Ausência de metástase para linfonodos regionais em exame histológico e resultado negativo no estudo molecular (RT-PCR).
pN0(mol+)	Resultado positivo do estudo molecular (RT-PCR), mas ausência de metástase para linfonodos regionais detectáveis histologicamente ou por IHC.
pN1	Micro-metástases ou; metástases em 1-3 linfonodo(s) axilar(es) ou; metástases para linfonodo(s) mamário(s) interno(s) detectado por biópsia de linfonodo(s) sentinela(s), mas não detectável(is) clinicamente.
pN1mi	Micro-metástases (maior que 0,2 mm e/ou maior que 200 células, mas nenhuma maior que 2,0 mm).
pN1a	Metástase em 1 a 3 linfonodo(s) axilare(s) e ao menos uma metástase maior que 2,0mm.
pN1b	Metástase em linfonodo(s) mamário(s) interno(s) com micro-metástases ou macro-metástases detectado(s) por biópsia de linfonodo(s) sentinela(s) mas não detectável clinicamente.[c]
pN1c	Metástase em 1 a 3 linfonodo(s) axilar(es) e em linfonodo(s) mamário(s) interno(s) com micro-metástases ou macro-metástases detectados por biópsia de linfonodos sentinelas, mas não detectável clinicamente.
pN2	Metástases em 4 a 9 linfonodos axilares ou em linfonodo(s) mamário(s) interno(s) clinicamente detectado[d] na ausência de metástases para linfonodos axilares.
pN2a	Metástases em 4 a 9 linfonodos axilares (pelo menos 1 aglomerado maior que 2,0mm).
pN2b	Metástases em linfonodo(s) mamário(s) interno(s) clinicamente detectável(is)[d]

	na ausência de metástases para linfonodos axilares.
pN3	Metástases em 10 ou mais linfonodos axilares ou; em linfonodos infraclaviculares (nível III axilar) ou; em linfonodo(s) mamário(s) interno(s) ipsilateral(is) na presença de um ou mais linfonodos axilares positivos nível I e II ou; em mais de três linfonodos axilares e em linfonodos mamários internos com micro ou macro-metástase detectados por biópsia de linfonodo sentinela mas não clinicamente detectável[c] ou; em linfonodos supraclaviculares ipsilaterais.
pN3a	Metástase em 10 ou mais linfonodos axilares (pelo menos um aglomerado maior que 2,0mm) ou linfonodos infraclaviculares (nível III axilar).
pN3b	Metástases em linfonodo(s) mamário(s) interno(s) ipsilateral(is) clinicamente detectáveis[d] na presença de um ou mais linfonodos axilares positivos ou; em mais de três linfonodos axilares e em linfonodos mamários internos com micro ou macro-metástases detectados por biópsia de linfonodos sentinelas mas não detectáveis clinicamente[c].
pN3c	Metástase em linfonodo(s) supraclavicular(es) ipsilateral(is)

H&E = Coloração por hematoxilina e eosina; IHC = Imunohistoquímica; ITC =células tumorais isoladas; RT-PCR = Reação em cadeia da polimerase com transcrição reversa
[a]Reproduzido com permissão do AJCC: Breast. In: Edge SB, Byrd DR, Compton CC, et al., eds.: AJCC Cancer Staging Manual. 7th ed. New York, NY: Springer, 2010, pp 347-76.
[b] Classificação baseada na dissecção dos linfonodos axilares com ou sem a biópsia de linfonodos sentinela. Classificação baseada unicamente na biópsia de linfonodos sentinelas sem subsequente dissecção de linfonodos axilares é designado SN (sentinel node), por exemplo, pNO (SN).
[c] "Não detectável clinicamente" é definido como aquele que não é detectável por estudos de imagem (exceto linfocitilografia) ou por exames clínicos.
[d] "Clinicamente detectável" é definido como aquele que é detectável por estudos de imagem (exceto linfocitilografia) ou por exames clínicos tendo características altamente suspeitas de malignidade ou uma macro-metástase patológica baseada em uma punção aspirativa por agulha fina com exame citológico.

Tabela 8. Classificação das metástases a distância (M)[a]

M0	Nenhuma evidência clínica ou radiográfica de metástases à distância.
cM0(i+)	Ausência de evidência clínica ou radiográfica de metástase à distância, mas presença molecular ou microscópica de células tumorais na circulação sanguínea, medula óssea, ou outro linfonodo não-regional menor que 0,2mm em paciente assintomáticos ou sinais de metástases.
M1	Metástase distante detectável clínica e radiograficamente e/ou histologicamente maior que 0,2mm.

[a]Reproduzido com permissão do AJCC: Breast. In: Edge SB, Byrd DR, Compton CC, et al., eds.: AJCC Cancer Staging Manual. 7th ed. New York, NY: Springer, 2010, pp 347-76.

COMITÊ DE ÉTICA

Universidade Estadual de Londrina

COMITÊ DE ÉTICA EM PESQUISA

COMITÊ DE ÉTICA EM PESQUISA ENVOLVENDO SERES HUMANOS
Universidade Estadual de Londrina/ Hospital Universitário Regional Norte do Paraná
Registro CONEP 268

Parecer PF Nº. 233/09 **CAAE Nº. 0179.0.268-09** **FOLHA DE ROSTO Nº. 294246**	Londrina, 20 de abril de 2010.

PESQUISADORA: **MARIA ANGELICA EHARA WATANABE**
CCB/DEPTO DE PATOLOGIA

Prezada Senhora:

O **"Comitê de Ética em Pesquisa Envolvendo Seres Humanos da Universidade Estadual de Londrina/ Hospital Universitário Regional Norte do Paraná"** (Registro CONEP 268)– de acordo com as orientações da Resolução 196/96 do Conselho Nacional de Saúde/MS e Resoluções Complementares, avaliou o projeto:

"ANÁLISE DA EXPRESSÃO DE GENES RELACIONADOS A CÉLULAS T REGULADORAS (TREGS) FOXP3+ EM PACIENTES COM CANCÊR DE MAMA"

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 relatório final da pesquisa.

Atenciosamente,

Profª. Dra. Alexandrina Aparecida Maciel

Coordenadora
Comitê de Ética em Pesquisa-CEP/UEL

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

UNIVERSIDADE
ESTADUAL DE LONDRINA

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

A – Informações sobre a pesquisa:

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 – Procedimentos do Estudo:

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 – Confidencialidade da Pesquisa

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

A amostra de sangue 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 nehuma compemsação 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) 3371-5728.

D – Consentimento livre esclarecido e informado:

Eu, _____, RG _____, declaro que estou de acordo com as informações contidas neste documento, fui devidamente esclarefcido 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: _____