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JULIE MASSAYO MAEDA ODA

**Polimorfismo T869C e expressão de TGF- β :
fator emergente no microambiente do
câncer de mama**

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JULIE MASSAYO MAEDA ODA

**Polimorfismo T869C e expressão de TGF- β :
fator emergente no microambiente do
câncer de mama**

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

Orientadora: Prof^ª. Dr^ª. Maria Angelica Ehara Watanabe

Co-Orientadora: Prof^ª. Dr^ª. Roberta Losi Guembarovski

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BANCA EXAMINADORA

Prof^a. Dr^a. Maria Angelica Ehara Watanabe
Universidade Estadual de Londrina

Prof^a. Dr^a. Eiko Nakagawa Itano
Universidade Estadual de Londrina

Prof^a. Dr^a. Regina Célia Poli-Frederico
Universidade Norte do Paraná

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"...E nunca considerem seu estudo como uma obrigação, mas sim como uma oportunidade invejável de aprender, sobre a influência libertadora da beleza no domínio do espírito, para seu prazer pessoal e para o proveito da comunidade à qual pertencerá o seu trabalho futuro."

ALBERT EINSTEIN

RESUMO

ODA, J.M.M. **Polimorfismo T869C e expressão de TGF- β : fator emergente no microambiente do câncer de mama.** Trabalho de Dissertação de Mestrado – Programa de Pós-Graduação em Patologia Experimental, Departamento de Ciências Patológicas, Centro de Ciências Biológicas (CCB) – Universidade Estadual de Londrina (UEL), 2010.

A família de polipeptídeos do fator de crescimento de transformação (TGF- β) é composta por citocinas que parecem exercer duas funções principais, homeostase do tecido e resposta à injúria tecidual. A imunopatologia associada à hiperativação da via do TGF- β 1 e na progressão tumoral é um dos principais motivos que têm atraído a atenção para este gene como um novo alvo terapêutico. CXCR4, um receptor transmembrana (sete- α -hélices) acoplado à proteína G é expresso em uma ampla variedade de tecidos e células-tronco órgão específico e é conhecido que está superexpressa em cânceres tendo função importante na invasão e metástase. A proposta deste estudo foi investigar o polimorfismo T869C do TGF- β e sua expressão em associação com a expressão de CXCR4 em pacientes com câncer de mama. DNA e RNA de 21 pacientes com câncer de mama foram analisados para o polimorfismo do TGF- β e expressão gênica dos genes TGF- β e CXCR4 por qRT-PCR. Em relação à frequência alélica, foi observado que o alelo C obteve uma distribuição de 0,43 e o alelo T uma distribuição de 0,56 e não houve diferença significativa na distribuição dos genótipos de acordo com as características clinicopatológicas. Os pacientes homozigotos CC apresentaram uma maior expressão de TGF- β , embora não significativa. As expressões relativas do RNAm de CXCR4 e TGF- β foram comparadas e uma correlação positiva foi observada ($p = 0,020$). Também houve uma associação entre a menor expressão de TGF- β com o aumento do tamanho do tumor ($p = 0,025$) $\rho(\text{rho} = 0,484)$, assim com o comprometimento de linfonodos ($p=0,033$) $\rho(\text{rho} = 0,400)$. Nossos resultados, incluindo a associação positiva entre o TGF- β e expressão de CXCR4 e a correlação tanto do tamanho do tumor quanto o comprometimento de linfonodos com a baixa expressão de TGF- β , sugere esse gene como um fator emergente no microambiente do câncer de mama.

Palavras Chaves: Câncer de Mama, polimorfismo T869C, TGF- β , CXCR4, PCR em Tempo Real.

ABSTRACT

ODA, J.M.M. **Polymorphism T869C and expression of TGF- β : emerging factor in breast cancer microenvironment.** Master Degree – Post-Graduate Program in Experimental Pathology, Department of Pathological Sciences, Biological Science Center (CCB) – University State of Londrina (UEL), 2010.

The transforming growth factor beta family (TGF- β) of polypeptides is a cytokine that appears to exert two major functions, tissue homeostasis and the response to tissue injury. The immunopathology associated with hyperactivation of the TGF- β 1 pathway in tumor progression is one of the main reasons that have attracted attention for this gene as a novel therapeutic target. CXCR4, a seven transmembrana G-coupled receptor protein is expressed on a wide variety of tissue and organ specific stem cells and it is known that is overexpressed in cancer and plays a role in invasion and metastasis. The propose of this study was to investigate the TGF- β T869C polymorphism and its expression correlated with CXCR4 expression in breast cancer patients. DNA and RNA from 21 breast cancer patients were analyzed for the TGF- β polymorphism and TGF- β and CXCR4 relative expression by qRT-PCR. Regarding the allele frequency it was observed that the allele C obtained a distribution of 0.43 and a T allele distribution of 0.56 and there was no significant difference in genotype distribution according to clinic pathological characteristics. The homozygous CC patients presented a higher TGF- β expression, although not significant. The relative mRNA expressions of CXCR4 and TGF- β were compared and a positive correlation was observed ($p= 0.020$). There was also an association between lower expression of TGF- β with increasing tumor size ($p = 0.025$) $\rho(\text{rho}= 0.484)$ and with lymph node involvement ($p = 0.033$) $\rho(\text{rho}= 0.400)$. Our results, including the positive correlation between TGF- β and CXCR4 expression and the correlation of tumor size as the lymph node involvement with low expression of TGF- β , suggests this gene as an emerging factor in the microenvironment of breast cancer.

Keywords: Breast Cancer, T869C polymorphism, TGF- β , CXCR4, real time PCR.

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LISTA DE ABREVIATURAS E SIGLAS

α	Alfa
3'UTR	(3' <i>UnTranslated Region</i>): Região 3' Não Codificadora
AgNO ₃	Nitrato de Prata
AJCC	(<i>American Joint Committee on Cancer</i>): Comitê Americano de Câncer
bp	(<i>base pair</i>): pares de base
CD	(<i>Cluster of Differentiation</i>): 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	<i>Cycle Threshold</i>
CXCL12	Quimiocina (família CXC) 12
CXCR4	Receptor de quimiocina (família CXC) 4
DEPC	Dietil Pirocarbonato
DNA	(<i>Desoxyribonucleic Acid</i>): Ácido Desoxirribonucléico
DNAc	Ácido Desoxirribonucléico Complementar
dNTP	Desoxirribonucleotideo trifosfato
E	Eficiência
EDTA	(<i>EthyleneDiamineTetraacetic Acid</i>): Ácido Dietilenoaminotetraacetato Dissódico
EHW	Equilíbrio de Hardy Weinberg
ER	(<i>Estrogen Receptor</i>): Receptor de Estrógeno
FD	Fator de Diluição
Foxp3	<i>Forkhead box P3</i>
GAPDH	Gliceraldeído 3-fosfato desidrogenase
h	Horas
HCL	Hospital do Câncer de Londrina

HU	Hospital Universitário
IL	Interleucina
INCA	Instituto Nacional de Câncer
kD	Kilodalton
Leu	Leucina
µg	Micrograma
µL	Microlitro
mg	Miligrama
min	Minutos
mL	Mililitro
mM	Milimolar
M-MLV	<i>Moloney Murine Leukemia Virus</i>
NCBI-NIH	<i>National Center for Biotechnology Information - National Institutes of Health</i>
NK	<i>Natural killer cell</i>
nm	Nanômetro
°C	Graus Celsius
PBMC	<i>(Peripheral Blood Mononuclear Cells): Células Mononucleares do Sangue Periférico</i>
PCR	Reação em Cadeia da Polimerase
PR	<i>(Progesterone Receptor): Receptor de Progesterona</i>
Pro	Prolina
qRT-PCR	<i>(Real Time quantitative Reverse Transcription PCR): PCR quantitativo em tempo real</i>
RFLP	<i>(Restriction Fragment Length Polymorphism): Análise de Restrição de Fragmentos Polimórficos</i>
RNA	Ácido ribonucléico

RNAM	Ácido Ribonucléico mensageiro
rpm	Rotações por minuto
RT-PCR	(<i>Reverse Transcription Polymerase Chain Reaction</i>): Reação em Cadeia da Polimerase via Transcriptase Reversa
s	Segundos
SDS	(<i>Sodium Dodecyl Sulfate</i>): Dodecil Sulfato de Sódio
SE	(<i>Standard Error</i>): Erro padrão
SNP	(<i>Single Nucleotide Polymorphism</i>): Polimorfismo de um Único Nucleotídeo
TCR	(<i>T Cell Receptor</i>): Receptor de Célula T
TGF- β	(<i>Transforming Growth Factor Beta</i>): Fator de Crescimento de Transformação beta
TMEPAI	(<i>Transforming Growth Factor-Beta Induced Transmembrane Protein</i>): Proteína Transmembrana Induzida pelo TGF- β
TNM	Tumor-Nódulo-Metástase (Sistema internacional de classificação de tumores)
Treg	(<i>Regulatory T cells</i>): Células T regulatórias
U	Unidade
UICC	União Internacional de Controle do Câncer
V	Volts
VEGF	(<i>Vascular Endothelial Growth Factor</i>): Fator de crescimento do endotélio vascular
XC	Xileno Cianol
%	Porcentagem

1 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 contribuindo para a iniciação, crescimento, invasividade do tumor e desenvolvimento de metástase (Yaquub e Aandahl, 2009). A interação entre os tumores e seu microambiente imunológico é complexa e difícil de decifrar e sua compreensão é de fundamental importância para o desenvolvimento de novos marcadores prognósticos e estratégias terapêuticas (Fridman et al, 2010).

A importância mundial do câncer é inquestionável, uma vez que é a segunda maior causa de mortes no mundo, seguida apenas de 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

No Brasil estima-se 49.240 novos casos de câncer de mama para 2010, com estimativa de risco de 49 casos para 100 mil mulheres (Brasil, 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 e 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 e Wittekind, 2002).

O ambiente hormonal também influencia o curso da doença (Briskin e O'Malley, 2010). Os cânceres de mama classificados pela expressão de receptores de estrógeno (ER) e progesterona (PR) 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 (Haslam e Shyamala 1981; Daniel et al. 1987; Haslam 1989). Postula-se que os fatores de risco estão intimamente associados aos tumores de mama ER+ e PR+ e pode envolver mecanismos relacionados à exposição de estrogênio e progesterona, enquanto que a etiologia do câncer de mama ER- e PR- pode ser independente da exposição hormonal (Potter et al, 1995; Huang et al, 2000; Enger et al, 2000; Yoo et al, 2001; Manjer et al, 2001).

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 cancerosas recrutam e ativam células inflamatórias que estimulam a progressão tumoral. Entretanto, as células inflamatórias também podem produzir citocinas que limitam o crescimento do tumor (Lin e Karin, 2007).

Citocinas

Citocinas liberadas em resposta à infecção, inflamação e na sinalização imunológica podem inibir o desenvolvimento e a progressão tumoral. Por outro lado, a ação das mesmas sobre células transformadas, pode levar ao crescimento tecidual, deficiência no mecanismo de apoptose, e ainda facilitar o processo de metástase (Dranoff, 2004). Crescentes evidências sugerem que citocinas, quimiocinas e fatores de crescimento são os principais agentes responsáveis na carcinogênese (Capone et al, 2010; Carpi et al, 2009).

As quimiocinas constituem uma grande família de citocinas estruturalmente homólogas responsáveis pela movimentação dos leucócitos, incluindo sua migração para locais de inflamação tecidual a partir do sangue.

Citocinas e quimiocinas (citocinas quimiotáticas) são proteínas solúveis produzidas por leucócitos e células não leucocitárias. Estes fatores solúveis influenciam em muitos aspectos da biologia leucocitária e do sistema imune. Estão envolvidos na proliferação celular, diferenciação, ativação e migração (Karupiah, 2003).

As quimiocinas são pequenas proteínas solúveis (8-14 kDa) que se ligam a um receptor acoplado à proteínas G para induzir uma resposta celular, usualmente direcionando migração ou quimiotaxia (Rot e von Andrian, 2004). Baseado na posição das duas cisteínas residuais N-terminais das quimiocinas, elas são classificadas em quatro grupos, conhecidos como CXC, CC, CX₃C e C (Baggiolini et al, 1997; Luster, 1998; Rossi e Zlotnik, 2000; Zlotnik e Yoshie, 2000). As duas principais famílias são a das quimiocinas CC, nas quais resíduos de cisteína são adjacentes, e a família CXC, na qual esses resíduos são separados por um aminoácido. Uma terceira família tem como característica estrutural a presença de duas cisteínas separadas por três aminoácidos (CX₃C). Finalmente, a quarta família possui uma única cisteína (família C). A nomenclatura das quimiocinas e seus receptores foi recentemente revisada com o objetivo de uniformizar o modo como eram citadas (IUIS/WHO, 2003).

As quimiocinas CXC se ligam e ativam seus respectivos receptores de quimiocinas em neutrófilos, linfócitos, células endoteliais e epiteliais. As quimiocinas CC atuam em muitas subpopulações de células dendríticas, linfócitos, macrófagos, eosinófilos, células *natural killer* (NK), mas não estimulam neutrófilos. As quimiocinas C e CX₃C são considerados as duas famílias de menores componentes. A família C é representada por duas quimiocinas, XCL1/linfotactina- α e XCL2/linfotactina- β , enquanto a família CX₃C contém somente um membro, o CX₃CL1/fractalina (Stievano et al, 2004). Existem aproximadamente 50 quimiocinas e somente 20 receptores de quimiocinas, portanto há uma considerável redundância neste sistema de interações entre ligante e receptor (Raman et al, 2007).

Uma variedade de citocinas, quimiocinas e fatores de crescimento são produzidos no ambiente tumoral por diferentes tipos celulares através de interações complexas e regulação de diferenciação, ativação, função e sobrevivência dos inúmeros tipos celulares. Esta interação entre citocinas, quimiocinas e fatores de crescimento 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 anti-tumoral e rejeição do tumor (Shurin et al, 2006).

CXCR4

Dentre as quimiocinas, o par mais ininteressante é o receptor de quimiocina CXC 4 (CXCR4) e seu ligante de quimiocina CXC 12 (CXCL12) (Liu et al, 2010). Os receptores de quimiocinas desempenham papéis relevantes no desenvolvimento da metástase, facilitando a disseminação tumoral em cada um dos eventos do processo, incluindo aderência das células tumorais ao endotélio, extravasamento para os vasos sanguíneos, colonização metastática, angiogênese, proliferação e proteção contra resposta do hospedeiro através da ativação de vias de sobrevivência celular (Kakinuma e Hwang, 2006; Singh et al., 2007).

O câncer de mama é caracterizado por um padrão metastático distinto, envolvendo linfonodos, medula óssea, fígado e pulmão. A migração das células tumorais e metastáticas tem muita semelhança com o tráfego de leucócitos. Recentemente verificou-se que células tumorais, como exemplo células do tumor de mama, podem ser guiadas através da circulação sanguínea até órgãos distantes que expressam quimiocinas específicas como a CXCL12, pois estas células possuem receptores específicos que possibilitam este evento. As células tumorais invadem estes órgãos, possibilitando a origem de tumores secundários (Muller et al., 2001).

Células de câncer de mama expressam altos níveis de CXCR4 (receptor da quimiocina CXCL12) funcionais, que podem direcionar a quimiotaxia e respostas invasivas (Muller et al., 2001). Os órgãos que apresentam expressão elevada de CXCL12 representam os sítios mais comuns de metástase no câncer de mama. Além disso, células metastáticas de linfonodos axilares e órgãos distantes como fígado e pulmão mostram forte expressão de CXCR4, sendo este aumento associado a uma baixa sobrevida em pacientes com câncer de mama (Luker e Luker, 2006).

Outra citocina envolvida nos cânceres é o fator de crescimento de transformação beta (TGF- β). É uma citocina dimérica expressa por diferentes tecidos (Blobe et al, 2000). Tem sido relatado que o TGF- β está superexpresso em muitos tumores, incluindo os de mama e parece estar relacionado à transformação e progressão do tumor (Friess et al, 1993; Tsushima et al, 1996; Comerci et al, 1996; Eder et al, 1996).

Portanto, a investigação sobre as interações entre o TGF- β e células no microambiente tumoral pode proporcionar uma melhor compreensão sobre a regulação e patogênese do câncer de mama.

TGF- β

O fator de crescimento de transformação beta (TGF- β) é um membro de uma ampla família de polipeptídeos multifuncionais secretados, que são potentes reguladores do crescimento celular, diferenciação e produção de matriz (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 contribui para a patogênese de diversas doenças, como as autoimunes (Cohen, 2003; Luethviksson e Gunnlaugsdottir, 2003) e carcinogênese (Tang et al., 2003; Tsuji et al., 2003).

TGF- β foi inicialmente identificado e nomeado baseado em sua habilidade de estimular o crescimento de fibroblastos em agar, mas atualmente é mais estudado como proteína inibitória do crescimento (Lee e Bae, 2002).

A função do TGF- β no sistema imune é a manutenção da tolerância via regulação da proliferação, diferenciação e sobrevivência dos linfócitos. Além disso, o TGF- β controla a iniciação e a resolução da resposta inflamatória através da regulação de quimiotaxia, ativação e sobrevivência de linfócitos, células *natural killer* (NK), células dendríticas, macrófagos, mastócitos e granulócitos. A atividade regulatória do TGF- β é modulada pelo estado de diferenciação e pela presença de citocinas inflamatórias e moléculas co-estimulatórias (Li et al, 2006). Esta citocina sinaliza sua ação através de receptores serina/treonina quinases e moléculas efetoras intracelulares conhecidas como Smads (Joshi e Cao, 2010).

O TGF- β é um biomarcador associado com a 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 TGF- β nas células e no microambiente tumoral tem influências positiva e negativa no desenvolvimento do cancer.

A família de proteínas do fator de crescimento de transformação beta (TGF- β) é altamente conservada evolutivamente (Zhang et al, 2006). São conhecidos três isoformas de TGF- β (TGF- β 1, TGF- β 2 e TGF- β 3), dos quais o TGF- β 1 é o mais abundante (Zheng, 2009).

O gene do TGF- β 1 está localizado no cromossomo 19q13 (Fujii et al, 1986). Muitos polimorfismos no gene TGF- β têm sido relatados (Cambien et al., 1996). Dois polimorfismos comumente estudados do TGF- β 1 são T869C (rs1800470; rs1982073; T29C; Leu10Pro), que está no exon 1 e pode levar a uma substituição de uma leucina para uma prolina no códon 10 e C-509T (rs1800469), que está em uma região promotora (Chang et al, 2008; Zheng, 2009; Yuan et al, 2009). Ambos os polimorfismos estão associados com um aumento no nível plasmático de TGF- β 1 (Grainger et al, 1999; Dunning et al, 2003). O polimorfismo T869C está associado à redução da sobrevida livre da doença (Shu et al, 2004; González-Zuloeta Ladd et al, 2007). Kirshner et al (2006) verificaram que indivíduos com o alelo prolina (alelo C) possuem aumento de 2,8 vezes na secreção de TGF- β comparada ao alelo leucina *in vitro*.

Estudos mostram que o TGF- β 1 está superexpresso em muitos tumores, incluindo os de mama e pode estar relacionado à transformação e progressão do tumor (Friess et al, 1993; Tsushima et al, 1996; Comerci et al, 1996; Eder et al, 1996). Os mecanismos relacionados à transformação e progressão são os seguintes: (1) a produção de TGF- β 1 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) TGF- β 1 pode promover o acúmulo de glicoproteínas da matriz extracelular e adesão protéica 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 TGF- β 1 pode aumentar a mobilidade celular e a produção de protease (Samuel et al, 1992); (4) superexpressão de uma proteína transmembrana induzida pelo TGF- β em muitos cânceres, a TMEPAI (*Transforming Growth Factor-Beta (TGF-Beta) Induced Transmembrane Protein*), e estudos sugerem que a TMEPAI converte o TGF- β de supressor de tumor para promotor; (5) recentemente, foi descoberto que o TGF- β induz a expressão de Foxp3(*Forkhead Box P3*) em células T CD4+CD25+Foxp3- naïve e as converte em células T regulatórias (Tregs) Foxp3+ (Chen et al, 2003; Zhang et al, 2006).

Embora as Tregs compreendam a maior porcentagem de células T CD4+ circulantes em pacientes com câncer de mama metastático e pacientes com recidiva após completa quimioterapia adjuvante, o *pool* sistêmico de Tregs parece ser independente do estágio da doença ou do tipo de tratamento (Rech et al, 2010).

Células T CD4+CD25+ que expressam Foxp3 (*Forkhead Box P3*) são reconhecidos como células T reguladoras profissionais e são instrumentos na indução e manutenção da tolerância imune (Sakaguchi, 2000; Shevach, 2002; Bluestone e Abbas, 2003; Powrie e Maloy, 2003; Fontenot e Rudensky, 2005; Schwartz, 2005; von Boehmer, 2005; Waldmann et al., 2006; Hill et al., 2007; Chen, 2009).

2 OBJETIVOS

2.1 *Objetivo Geral*

Analisar o polimorfismo T869C e a expressão da citocina TGF- β 1 e do receptor de quimiocina CXCR4 no microambiente do câncer de mama.

2.2 *Objetivos Específicos*

- Avaliar os dados clinicopatológicos das pacientes doadoras de tecido mamário;
- Estimar a frequência do genótipo do polimorfismo T869C do TGF- β 1;
- Comparar o polimorfismo T869C do TGF- β 1 com os dados clinicopatológicos;
- Comparar a frequência alélica com as características clinicopatológicas das pacientes;
- Determinar a expressão gênica do TGF- β 1 e CXCR4 no tecido;
- Relacionar a expressão gênica do TGF- β 1 com os dados clinicopatológicos;
- Confrontar o polimorfismo T869C do TGF- β 1 com a expressão gênica desta mesma citocina;
- Correlacionar a expressão gênica do TGF- β 1 com a expressão gênica do receptor de quimiocina CXCR4 nos tecidos.

3 MATERIAIS E MÉTODOS

3.1 Consentimento Livre e Esclarecido

Este projeto foi aprovado pelo Comitê de Ética em Pesquisa Envolvendo Seres Humanos – Universidade Estadual de Londrina (CEP/UEL N° 233/09), o qual está de acordo com a Comissão Nacional de Ética em Pesquisa (CONEP-CAAE - 0278.0.268.000-06) e com a resolução 196/96 – Conselho Nacional da Saúde (CNS). Todas as participantes do projeto assinaram o Termo de Consentimento Livre e Esclarecido (vide Apêndice).

3.2 Seleção de Amostras

O convite as pacientes com diagnóstico de câncer de mama para participarem deste projeto de pesquisa ocorreu durante o atendimento clínico destas pacientes nos serviços especializados e no Hospital do Câncer de Londrina. As atividades de pesquisa foram realizadas no Laboratório de Genética Molecular e Imunologia, Departamento de Ciências Patológicas, Centro de Ciências Biológicas da Universidade Estadual de Londrina.

Os diagnósticos, os dados clínicos e a imunohistoquímica foram fornecidos pelo Departamento de Anatomia Patológica do Hospital do Câncer de Londrina (ICL) (vide Apêndice) e pelos Laboratórios de Análises Histopatológicas Micropar e Preventivo ambos em Londrina-PR.

O estudo consistiu em um grupo de amostras de tecido de 21 pacientes, entre 40-76 anos diagnosticadas com câncer de mama, submetidas à cirurgia de mama.

3.3 Estadiamento do câncer de mama segundo UICC

O critério utilizado para a classificação dos tumores envolvidos no presente estudo foi o criado pela União Internacional de Controle do Câncer (UICC), denominado Sistema Tumor-Nódulo-Metástase (TNM). O estadiamento clínico é importante, pois permite estabelecer a extensão e a gravidade da doença, planejar o tratamento, dar o prognóstico, ou seja, prever a evolução das enfermidades, e, finalmente, agrupar os casos para estudo e pesquisa (INCA/MS, 2007).

O Sistema Tumor-Nódulo-Metástase (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).

Após a avaliação destes fatores, os casos são classificados em estádios que variam de I a IV graus crescentes de gravidade da doença (INCA/MS, 2007). Esta classificação aplica-se apenas aos carcinomas, sendo indispensável à 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).

Os quadros a seguir sintetizam as classificações conforme o tamanho do tumor (T), comprometimento nodular (N) e metástases (M), além de agrupar as diversas combinações possíveis (INCA/MS, 2007).

TAMANHO DO TUMOR (T)

<ul style="list-style-type: none"> • Tx - tumor não pode ser avaliado
<ul style="list-style-type: none"> • T0 - não há evidência de tumor primário • Tis - carcinoma <i>in situ</i> • T1 - tumor com até 2 cm em sua maior dimensão • T1 mic - carcinoma microinvasor (até 1 mm) • T1a - tumor com até 0,5 cm em sua maior dimensão • T1b - tumor com mais de 0,5 e até 1 cm em sua maior dimensão • T1c - tumor com mais de 1 cm e até 2 cm em sua maior dimensão • T2 - tumor com mais de 2 e até 5 cm em sua maior dimensão • T3 - tumor com mais de 5 cm em sua maior dimensão • T4 - qualquer T com extensão para pele ou parede torácica

- T4a - extensão para a parede torácica
- T4b - edema (incluindo *peau d'orange*), ulceração da pele da mama, nódulos cutâneos satélites na mesma mama
- T4c - associação do T4a e T4b
- T4d - carcinoma inflamatório

Observações:

- a. O comprometimento do músculo grande peitoral não caracteriza T4.
- b. Presença de retração da pele ou papila não interfere no estadiamento.

LINFONODOS REGIONAIS (N)

- Nx - Os linfonodos regionais não podem ser avaliados
- N0 - Ausência de metástase
- N1 - Linfonodo(s) homolateral(is) móvel(is) comprometido(s)
- N2 - Metástase para linfonodo(s) axilar(es) homolateral(is), fixos uns aos outros ou fixos a estruturas vizinhas ou metástase clinicamente aparente somente para linfonodo(s) da cadeia mamária interna homolateral
- N2a - Metástase para linfonodo(s) axilar(es) homolateral(is) fixo(s) uns aos outros ou fixos à estruturas vizinhas
- N2b - Metástase clinicamente aparente somente para linfonodo(s) da cadeia mamária interna homolateral(is) em evidência clínica de metástase axilar
- N3 - Metástase para linfonodo(s) infraclavicular(es) homolateral(is) com ou sem comprometimento do(s) linfonodo(s) axilar(es), ou para linfonodo(s) da mamária interna homolateral clinicamente aparente na presença de evidência clínica de metástase para linfonodo(s) axilar(es) homolateral(is), ou metástase para linfonodo(s) supraclavicular(es) homolateral(is) com ou sem comprometimento do(s) linfonodo(s) axilar(es) ou da mamária interna
- N3a - Metástase para linfonodo(s) infraclavicular(es) homolateral(is)
- N3b - Metástase para linfonodo(s) da mamária interna homolateral e para linfonodo(s) axilar(es)
- N3c - Metástase para linfonodo(s) supraclavicular(es) homolateral(is)

Observação:

Clinicamente aparente é definido como detectado por estudos de imagem (exceto linfocintigrafia), pelo exame clínico ou pelo diagnóstico patológico macroscópico.

METÁSTASES (M)

- Mx metástase à distância não pode ser avaliada
- M0 ausência de metástase à distância
- M1 presença de metástase à distância (incluindo LFN supraclaviculares)

ESTADIAMENTO TNM DO CÂNCER DE MAMA POR AGRUPAMENTOS

Estádio 0	Tis N0 M0
Estádio I	T1 N0 M0
Estádio II A	T0 N1 M0
	T1 N1 M0
Estádio II B	T2 N0 M0
	T2 N1 M0
Estádio III A	T3 N0 M0
	T0 N2 M0
	T1 N2 M0
	T2 N2 M0
Estádio III B	T3 N1 M0
	T3 N2 M0
	T4 N0 M0
Estádio III C	T4 N1 M0
	T4 N2 M0
Estádio III C	Tqq N3 M0*
Estádio IV	Tqq Nqq M1*

Fonte: UICC, 2002; *qq = qualquer

3.4 Obtenção de células do tecido mamário

O tecido mamário tumoral e saudável pós-cirúrgico de pacientes com câncer de mama, previamente separados pelo anatomopatologista, foram homogeneizados por pinçamentos sucessivos em solução salina estéril e posteriormente centrifugados a 2000 rpm para obtenção das células do tecido. Adicionou-se então 600 µL de TRIzol-LS (Invitrogen, São Paulo, SP, Brasil) a fim de preservar a integridade do RNA, e as amostras foram mantidas em *freezer* - 20°C até a extração de RNA.

3.5 Análise Molecular

3.5.1 Extração de DNA

O DNA genômico foi obtido a partir de leucócitos do sangue periférico das pacientes com câncer de mama. A técnica utilizada para a extração do DNA consiste basicamente no

rompimento enzimático de membranas celulares, eliminação de proteínas e ácidos graxos por ação de solventes orgânicos e precipitação do DNA com etanol de acordo com a técnica descrita por Miller et al. (1988) e Kirby (1990), com algumas modificações.

Para algumas amostras foram necessárias extração de DNA a partir de blocos de tecido mamário previamente fixado em formalina tamponada e incluídos em parafina, cujo procedimento de extração foi realizado baseado no protocolo descrito por Isola et al (1994).

As amostras de DNA foram quantificadas por espectrofotometria no aparelho UV-1650PC (Shimadzu, Kyoto, Japan) nos comprimentos de onda de 260 nm e 280 nm. A concentração do DNA em ng/μL foi calculada utilizando-se $\text{ng}/\mu\text{L} = \text{Abs}_{260} \times \text{FD} \times 50$, considerando-se que uma unidade de absorbância a 260 nm equivale a 50 μg DNA/mL, e FD o fator de diluição. O grau de pureza em relação à contaminação por proteínas foi avaliado pela razão entre as absorbâncias nos comprimentos de 260 nm e 280 nm (260/280). Amostras de DNA que apresentaram relações de absorbâncias maiores ou iguais a 1,7 ($260/280 \geq 1,7$) foram utilizadas nos experimentos (White e De Lucca, 1977), pois são consideradas com baixas concentrações de proteínas. A integridade do DNA foi analisada rotineiramente por uma eletroforese a 100 V, em gel de agarose 1% (Figura 1).

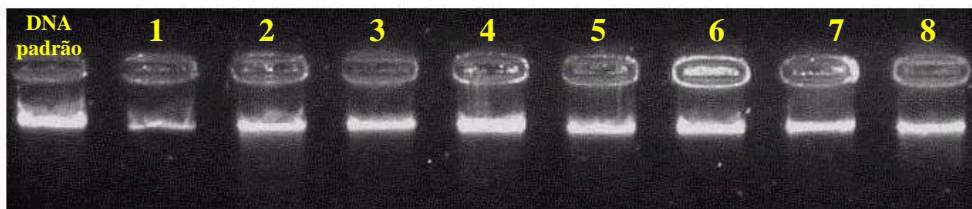


Figura 1 Integridade do DNA. DNA obtido do sangue periférico foram visualizados em gel de agarose 1% em presença de brometo de etídio. Foi aplicado 5 μL de DNA e 5 μL do corante XC (Xileno Cianol). DNA padrão corresponde ao DNA com concentração conhecida (100ng/μL) e as amostras de 1-8 correspondem ao material genético extraído do *buffy coat*.

3.5.2 Análise do polimorfismo TGF-β T869C

Aproximadamente 200 ng de DNA foram amplificados pela reação em cadeia da polimerase (PCR) com *primers* específicos para o polimorfismo do TGF-β sintetizados de acordo com a sequência do *GenBank* NG_013364.1.

TGF- β (T):TGF- β (T) sense 5'- GGG CTG CGG CTG CTG CT -3'TGF- β (T) antisense 5'- GTA GTC GGC CTC AGG CTC GG -3'**TGF- β (C):**TGF- β (C) sense 5'- CTC CAC CAC TGC GCC CTT CT -3'TGF- β (C) antisense 5'- AGC AGC GGT AGC AGC AGC G -3'

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5581 tcctagacct tttctcctcc aggagacgga tctctctccg acctgccaca gatcccctat
5641 tcaagaccac ccaccttctg gtaccagatc ggcgccatct aggttatttc cgtgggatac
5701 tgagacaccc ccggtccaag cctcccctcc accactgcgc ccttctccct gaggacctca
5761 gctttccctc gaggccctcc taccttttgc cgggagacct ccagccctg caggggcggg
5821 gcctccccac cacaccagcc ctgttcgcgc tctcggcagt gccggggggc gccgcctccc
5881 ccatgccgcc ctccgggctg cggtctctgc cgtctctgct accgctgctg tggctactgg
5941 tgctgacgcc tggccggccg gccgcgggac tatccacctg caagactatc gacatggagc
6001 tgggtgaagcg gaagcgcacg gaggccatcc gcgccagat cctgtccaag ctgctgctcg
6061 ccagccccc gagccagggg gaggtgccgc ccggcccgt gcccgagcc gtgctcgccc
6121 tgtacaacag caccgcgac cgggtggccg gggagagtgc agaaccggag cccgagcctg
6181 aggccgacta ctacgccaag gaggtcaccg gcgtgctaat ggtggaaacc cacaacggtg
6241 agctcggagg ggcaggggag ccgggagggg ggccccagc ggcgcgccga gtgcccggggc

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As amostras foram amplificadas utilizando *buffer plus* com 1,25 U de Taq polimerase (InvitrogenTM, Carlsbad, California, USA). As condições da reação de amplificação estão apresentadas no quadro abaixo (Tabela 1), e foram realizadas em termociclador (PCR-Sprint Hybaid - Guelph, Ontario, Canada). Inicialmente, uma etapa de desnaturação foi constituída por 10 minutos a 95 °C, seguida de 35 ciclos de 94 °C por 1 minuto, 69 °C (para o alelo T) e 65°C (para o alelo C) por 1 minuto e 72°C por 1 minuto, com extensão final de 5 minutos a 72 °C.

Tabela 1 Condições da reação de amplificação do polimorfismo do TGF- β T869C.

TGF- β (T)		TGF- β (C)	
Reagentes	Volume	Reagentes	Volume
H ₂ O Milli-Q q.s.p.	11,25 μ L	H ₂ O Milli-Q q.s.p.	11,50 μ L
Buffer 10X *	2,5 μ L	Buffer 10X *	2,5 μ L
dNTP 1,25 mM *	2,0 μ L	dNTP 1,25 mM *	2,0 μ L
Primer Sense 2,5 μ M *	1,5 μ L	Primer Sense 2,5 μ M *	1,5 μ L
Primer Anti-Sense 2,5 μ M *	1,5 μ L	Primer Anti-Sense 2,5 μ M *	1,5 μ L
MgCl ₂ 50 mM *	1,0 μ L	MgCl ₂ 50 mM *	0,75 μ L
Taq (1:10) *	0,5 μ L	Taq (1:10) *	0,5 μ L
DNA Template	2,5 μ L	DNA Template	2,5 μ L
Total	25,00 μL	Total	25,00 μL

*INVITROGEN *Life Technologies* – Brasil.*INVITROGEN *Life Technologies* – Brasil.

Os fragmentos amplificados de 297 pb para TGF- β (T) e 204 pb para TGF- β (C) foram analisados por eletroforese em gel de poliacrilamida (10%) e corados com Nitrato de Prata (AgNO₃).

3.5.3 Extração do RNA do tecido mamário

As células do tecido mamário foram homogeneizadas em TRIzol-LS (Invitrogen, São Paulo, SP, Brasil) para obtenção de RNA, de acordo com as instruções do fabricante. O RNA obtido foi ressuspensionado em água tratada com Dietil Pirocarbonato (DEPC; LGC Biotecnologia, São Paulo, Brasil) estéril, livre de RNases, DNases e resíduos de proteínas.

As amostras foram quantificadas por espectrofotometria no aparelho *BioMate™ 3 Series Spectrophotometer* (Thermo Electron Scientific Instrument Corporation, USA) no comprimento de onda de 260 nm considerando-se que uma unidade de absorbância a 260 nm equivale a 40 μ g RNA/mL. A leitura também foi realizada a 280 nm. O grau de pureza em relação à contaminação por proteínas foi medido através da razão entre as absorbâncias nos comprimentos de 260 nm e 280 nm (260/280). Apenas as amostras de RNA que apresentarem relações de absorbâncias maiores ou iguais a 1,9 (260/280 \geq 1,9) foram utilizadas nos experimentos.

Antes dos ensaios com RNAm de CXCR4 e TGF- β , foi realizada a validação das amostras de RNA humano e a qualidade de DNAc foram avaliadas através de RT-PCR para o gene da β -actina. Todas as amostras de RNA apresentaram quantidades detectáveis de RNAm para β -actina e não demonstraram degradação. Não houve contaminação por DNA genômico, uma vez que todos os produtos amplificados apresentaram um único fragmento correspondente a 353 pb (Figura 2).

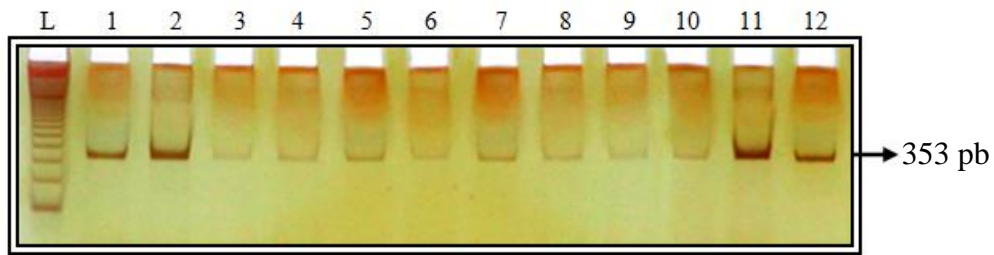


Figura 2 Expressão do RNAm de β -actina em amostras tumorais. Análise da integridade do RNA e da viabilidade do DNAc, e verificação da presença de DNA genômico. A amplificação do fragmento de 353 pb indica presença de RNAm β -actina. L – ladder de 100 bp (Invitrogen).

Os iniciadores utilizados para a amplificação foram obtidos de acordo com o GenBank *Accession number*: BC014861, como descrito por Amarante et al. (2005). A reação de PCR para β -actina foi submetida ao termociclador MG96 - *Biocycler* (importado por Biosystems, Curitiba PR, Brasil) utilizando-se: 20 mM Tris-HCl pH 8.4, 50 mM KCl, 1,5 mM MgCl₂, 200 μ M dNTP e 0.5 U de Taq polimerase. Inicialmente, uma etapa de desnaturação foi constituída por um minuto à 94 °C, seguida de 35 ciclos de 94 °C por 30 segundos, 55 °C por 30 segundos e 72 °C por 1 minuto, com extensão final de 10 minutos a 72 °C. O fragmento amplificado de β -actina de 353 pares de bases foi analisado por eletroforese em gel de poliacrilamida a 10% corado com Nitrato de Prata (AgNO₃).

3.5.4 Reação de Transcriptase Reversa (RT-PCR)

A síntese de DNAc foi realizada a partir de 7 μ L ou 500ng de RNA com 2,5 μ M de primer oligo d(T) e 1U de transcriptase reversa *Moloney Murine Leukemia Virus Reverse Transcriptase* (M-MLV RT; Invitrogen™, USA) a 42°C por 60 minutos. A reação foi efetuada em tampão específico (50 mM Tris-HCl pH 8,3; 75mM KCl; 1,5 mM MgCl₂ e 1,25 mM de dNTP). Todas as reações de síntese de DNAc foram realizadas em termociclador PCR *Sprint ThermoHybaid* (BioSystems, Guelph, Canada).

A quantidade de DNAc utilizada para a reação de PCR em tempo real foi previamente determinada através de uma curva na qual foi realizada diluições de 1:8, 1:10, 1:20, 1:40 e 1:80 do RNA obtido. A escolha foi baseada nas melhores eficiências e médias de CT (*Cycle Threshold*) das duplicatas.

3.5.5 PCR Quantitativo em Tempo Real (qRT-PCR)

Para o presente estudo, foi utilizado o gene GAPDH (gliceraldeído-3-fosfato desidrogenase) como gene constitutivo e os genes de interesse foram o CXCR4 e o TGF- β . A reação foi realizada em duplicata para cada amostra de DNAc utilizada e a temperatura de anelamento foi de 54°C.

A reação de RT-PCR quantitativa foi realizada a partir da diluição de 1:20 de DNAc em um volume final de 20 μ L, utilizando o fluoróforo Platinum[®]SYBR Green qPCR SuperMix UDG (Invitrogen TM) e 0.25 μ M dos iniciadores descritos na Tabela 2. A reação de PCR iniciou-se por uma etapa de desnaturação a 95°C por 2 minutos, seguida por 40 ciclos de 95°C por 30s, 54°C por 30s e 72°C por 30s no sistema de detecção Chromo4[™] Real Time PCR Detection (Bio-Rad, Hercules, USA). A análise da curva de *melting* foi sempre realizada no final da reação para verificar artefatos como dímeros de *primers* e contaminação. Além disso, em todos os experimentos, controles negativos apropriados (sem material genético) foram submetidos ao mesmo procedimento para excluir ou detectar qualquer eventual contaminação.

Tabela 2 Condições da Reação de RT-PCR Quantitativa

Gene alvo	GenBank Acession Number	Iniciador	Sequência	Temperatura de <i>Melting</i> (T°C)
CXCR4 mRNA	AF025375	<i>foward</i>	5' TCTACTCCATCATCTTCTTTA 3'	80.5
		<i>reverse</i>	5' ACGTTGGCAAAGATGAAGGTC 3'	
TGF β mRNA	NM_000660	<i>foward</i>	5' GTC GGG AGA AGA GGA AAA AAA 3'	79.5
		<i>reverse</i>	5' GGC AAA GGG AGG CGG TC 3'	
GAPDH mRNA	NM_002046	<i>foward</i>	5' GAA GGT GAA GGT CGG A 3'	80.5
		<i>reverse</i>	5' GGG TCA TTG ATG GCA AC 3'	

* Temperatura de anelamento = 54°C

3.5.6 Cálculo para expressão do RNAm

De acordo com Pfaffl (2001) pequenas diferenças nas eficiências das reações de RT-PCR quantitativo podem acarretar uma grande diferença na quantidade de produto final. Portanto, a expressão do gene de cada amostra foi calculada através da fórmula abaixo considerando as eficiências.

$$R = \frac{E_{\text{alvo}} (CT_{\text{normal}} - CT_{\text{ca.mama}})}{E_{\text{constitutivo}} (CT_{\text{normal}} - CT_{\text{ca.mama}})}$$

E = Eficiência

CT = *Cycle Threshold*

Obs: O CT_{valor} serve como uma ferramenta para calcular a quantidade inicial de DNAc em cada amostra. Este valor corresponde ao ciclo no qual a primeira fluorescência é detectada. O ótmo da configuração do *threshold* depende da reação química utilizada para a realização do PCR.

O valor de E representa as eficiências tanto do gene alvo quanto do constitutivo. Para a obtenção do E de determinado gene expresso, foi calculada a média das eficiências das duplicatas de cada amostra, assim como para a eficiência do gene constitutivo (E_{GAPDH}). Os valores de CT (*Cycle Threshold*) inseridos na fórmula representam uma média dos valores de CT das duplicatas de cada paciente para cada gene.

Para as amostras de tecido, o cálculo foi realizado comparando-se a eficiência e o CT do gene alvo do tecido mamário tumoral em relação ao tecido mamário normal (periférico ao tumor) da mesma paciente.

3.6 *Análise Estatística*

As análises estatísticas foram realizadas utilizando-se o programa SPSS Statistics 17.0 (SPSS inc., Chicago, Illinois, USA). Para análise do genótipo relacionada à expressão gênica e às variáveis clinicopatológicas foi utilizado o teste de Kruskal Wallis. Para análise da expressão relativa de RNAm foi utilizado o teste de Kruskal-Wallis e para verificar a correlação entre a expressão de TGF- β com as características clinicopatológicas foi utilizado o teste de Correlação de Pearson. Para todos os dados o nível de significância adotado foi de $p < 0,05$.

4 RESULTADOS

4.1 Características Clinicopatológicas

A faixa etária das pacientes envolvidas neste estudo variou de 40 a 76 anos, sendo que a maior incidência de câncer foi observada no grupo de mulheres com idade superior a 60 anos (42,86%, 09/21), como pode ser observado na Tabela 3.

A maioria das pacientes apresentou carcinoma ductal invasivo (95,24%, 20/21), de acordo com os critérios clínicos estabelecidos pelo *Union of International Control of Cancer* (UICC, 2002) e dentre elas, os tumores de estadiamento II e III (85,71%; 18/21) foram os mais predominantes, enquanto que apenas 14,28% (03/21) apresentaram tumores de estadiamento I ou IV. O tamanho do tumor predominante entre as pacientes variou de 2.1 – 5.0 cm, o que corresponde a cerca de 57,10% (12/21). A presença dos receptores de estrógeno (61,90% - 13/21) e progesterona (61,90% - 13/21) foi verificada na maioria das pacientes, e, todas as pacientes receptor de progesterona positiva foram também receptor de estrógeno positiva (Tabela 3).

4.2 Análise do polimorfismo TGF- β T869C

A frequência do genótipo de TGF- β foi avaliada através da PCR, a partir de amostras de DNA. A análise da eletroforese dos fragmentos amplificados permitiu a visualização de 3 diferentes genótipos: CC, CT e TT (Figura 3).

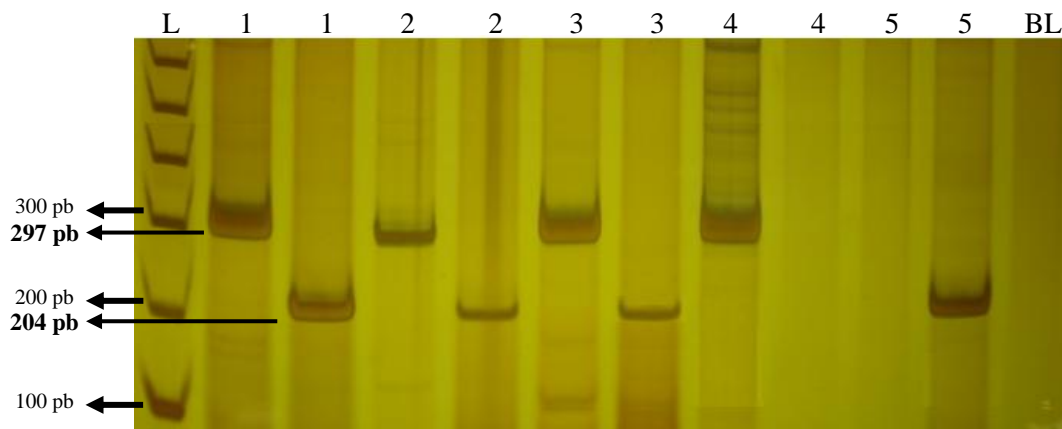


Figura 3 Perfil eletroforético do polimorfismo TGF- β T869C. Gel de poliacrilamida 10% (v/v) corado com nitrato de prata. L representa o marcador de peso molecular ladder de 100bp, as amostras de 1-5 apresentam

amplificação de 297bp para o alelo T e 204bp para o alelo C e BL corresponde ao blank (controle negativo). A amostra 1, 2 e 3 possuem genótipo CT, a amostra 4 genótipo TT e a amostra 5 genótipo CC.

O genótipo CC foi observado em 9,52% (02/21), o genótipo CT em 61,91% (13/21) e o genótipo TT em 28,57% (06/21) das pacientes (Figura 4). Em relação à frequência alélica, foi observado que o alelo C obteve uma distribuição de 0,43 e o alelo T uma distribuição de 0,56.

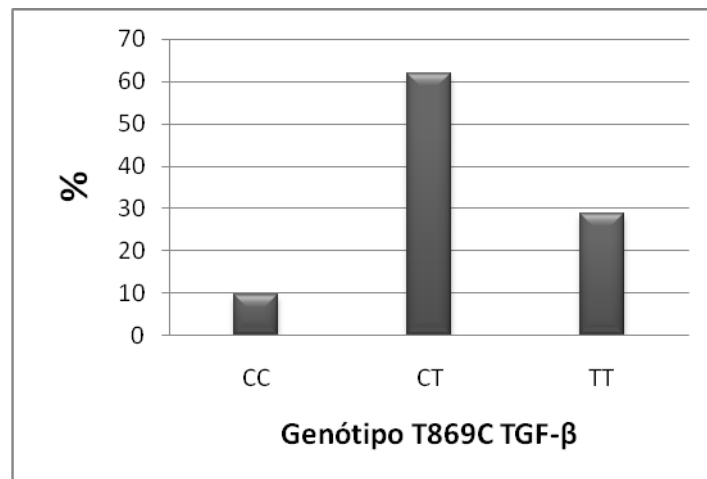


Figura 4 Distribuição genotípica do polimorfismo TGF- β T869C. Genótipo CC foi observado em 9,52% das pacientes, o genótipo CT foi encontrado em 61,91%, enquanto que o genótipo TT foi observado em 28,57% das pacientes.

Não foi observada diferença significativa na distribuição genotípica do TGF- β T869C de acordo com as características clinicopatológicas avaliadas, como faixa etária ($p=0,224$), a expressão de receptor de estrógeno ($p=0,092$), expressão de receptor de progesterona ($p=0,092$), classificação histológica ($p=0,287$), tamanho do tumor ($p=0,423$) e estadiamento ($p=0,479$) como demonstrado na Tabela 3.

Também não foi observada diferença significativa entre a frequência alélica do TGF- β com as características clinicopatológicas das pacientes, como faixa etária ($p=0,157$), a expressão de receptores hormonais ($p=0,495$), classificação histológica ($p=0,490$), tamanho do tumor ($p=0,495$) e estadiamento ($p=0,317$).

Tabela 3 Características clinicopatológicas das pacientes com câncer de mama relacionada ao polimorfismo TGF- β T869C.

		Numero de indivíduos N (%) (n=21)	Genótipo TGF- β		
			CC	CT	TT
			N(%)	N (%)	N (%)
Idade (anos)	< 40	1 (4,76)	0 (0,00)	0 (0,00)	1 (4,76)
	41 - 50	4 (19,05)	0 (0,00)	2 (9,52)	2 (9,52)
	51 – 60	7 (33,33)	1 (4,76)	4 (19,05)	2 (9,52)
	> 60	9 (42,86)	1 (4,76)	7 (33,33)	1 (4,76)
Receptor de Estrógeno (ER) e Progesterona (PR)	Positivo	13 (61,90)	1 (4,76)	6 (28,57)	6 (28,57)
	Negativo	6 (28,57)	1 (4,76)	5 (23,81)	0 (0,00)
	Desconhecido	2 (9,52)	0 (0,00)	2 (9,52)	0 (0,00)
Classificação Histológica^a	CDI	20 (95,24)	2 (9,52)	13 (61,90)	5 (23,81)
	CLI	1 (4,76)	0 (0,00)	0 (0,00)	1 (4,76)
Tamanho do Tumor	0 – 2.0	8 (38,10)	0 (0,00)	7 (33,30)	1 (4,80)
	2.1 – 5.0	12 (57,10)	2 (9,50)	5 (23,80)	5 (23,80)
	> 5.1	1 (4,80)	0 (0,00)	1 (4,80)	0 (0,00)
Estadiamento do Tumor	I	2 (9,52)	0 (0,00)	1 (4,76)	1 (4,76)
	II	13 (61,90)	2 (9,52)	7 (33,33)	4 (19,05)
	III	5 (23,81)	0 (0,00)	4 (19,05)	1 (4,76)
	IV	1 (4,76)	0 (0,00)	1 (4,76)	0 (0,00)

^a CDI – Carcinoma Ductal Invasor; CLI – Carcinoma Lobular Invasor; Especial – Adenocarcinoma

Todas as pacientes positivas para receptor de estrógeno foram também positivas para receptor de progesterona. Embora estatisticamente não significante, a Figura 5a mostra uma tendência de associação do genótipo TT em homozigose com receptor positivo para estrógeno e progesterona. A Figura 5b mostra que a maioria das pacientes que tinha tamanho aumentado de tumor era hormônio dependente.

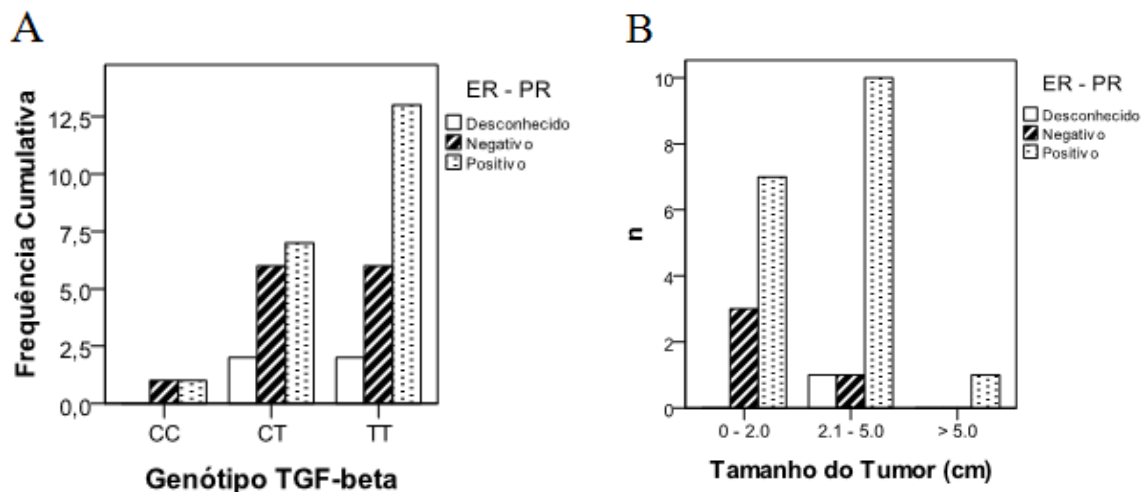


Figura 5 Avaliação relacionada ao receptor hormonal. a) Distribuição genotípica em relação aos receptores hormonais (estrógeno e progesterona) das pacientes que doaram o tecido mamário tumoral e respectivo tecido saudável. 28,57% das pacientes apresentaram ER+ e PR+ e pertenciam ao genótipo TT. (ER= Receptor de Estrógeno e PR= Receptor de Progesterona). *Kruskal-Wallis Test*, $p= 0,092$. Figura 5b. Distribuição dos receptores hormonais em relação ao tamanho do tumor. *Kruskal-Wallis Test*, $p=0,727$.

Embora a ocorrência do genótipo CT tenha sido prevalente dentre as pacientes, grande parte das mulheres com genótipo TT em homozigose apresentaram receptores hormonais positivos e a predominância do tamanho do tumor foi de 2.1 – 5.0 cm (Figura 5).

Houve predominância do estadiamento II (61.90%) entre as pacientes, das quais 33,33% possuíam genótipo CT e 19,05% genótipo TT e a maioria das pacientes de genótipo TT apresentou tumor com tamanho variando entre 2.1 – 5.0 cm.

4.3 Análise quantitativa da expressão gênica- RNAm

Foi realizada uma curva de diluição com várias concentrações de DNAC (1:8, 1:10, 1:20, 1:40 e 1:80) para determinar a quantidade ideal de material a ser administrada no PCR em Tempo Real. A escolha foi baseada nas melhores eficiências e médias de CT (*Cycle Threshold*) das duplicatas, conforme pode ser observado na Figura 6.

As análises de expressão relativa de RNAm, foram sempre comparadas entre o tecido tumoral mamário e o tecido periférico mamário, não tumoral, da mesma paciente, criteriosamente selecionada pelo patologista. Os cálculos foram realizados segundo Pfaffl, 2001, levando-se em conta as eficiências das reações.

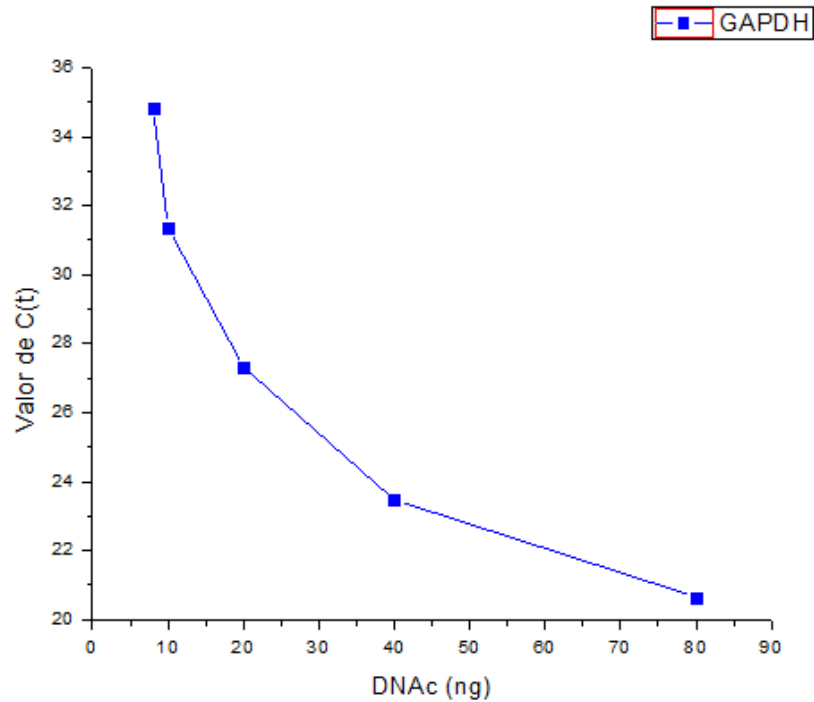


Figura 6 Curva de diluição do gene constitutivo GAPDH. Foi realizada uma curva de diluição com várias concentrações de DNAc (1:8, 1:10, 1:20, 1:40 e 1:80) para determinar a quantidade ideal de material a ser administrada no *Real Time PCR*. A escolha foi baseada nas melhores eficiências e médias de CT (*Cycle Threshold*) das duplicatas.

A análise da curva de *melting*, cujo protocolo foi descrito em materiais e métodos, revelou a presença de somente um pico correspondente aos fragmentos de GAPDH, na temperatura de 80.5°C, CXCR4, na temperatura de 80.5°C e de TGF- β a 79.5°C, confirmando a especificidade da amplificação, como ilustrado na Figura 7.

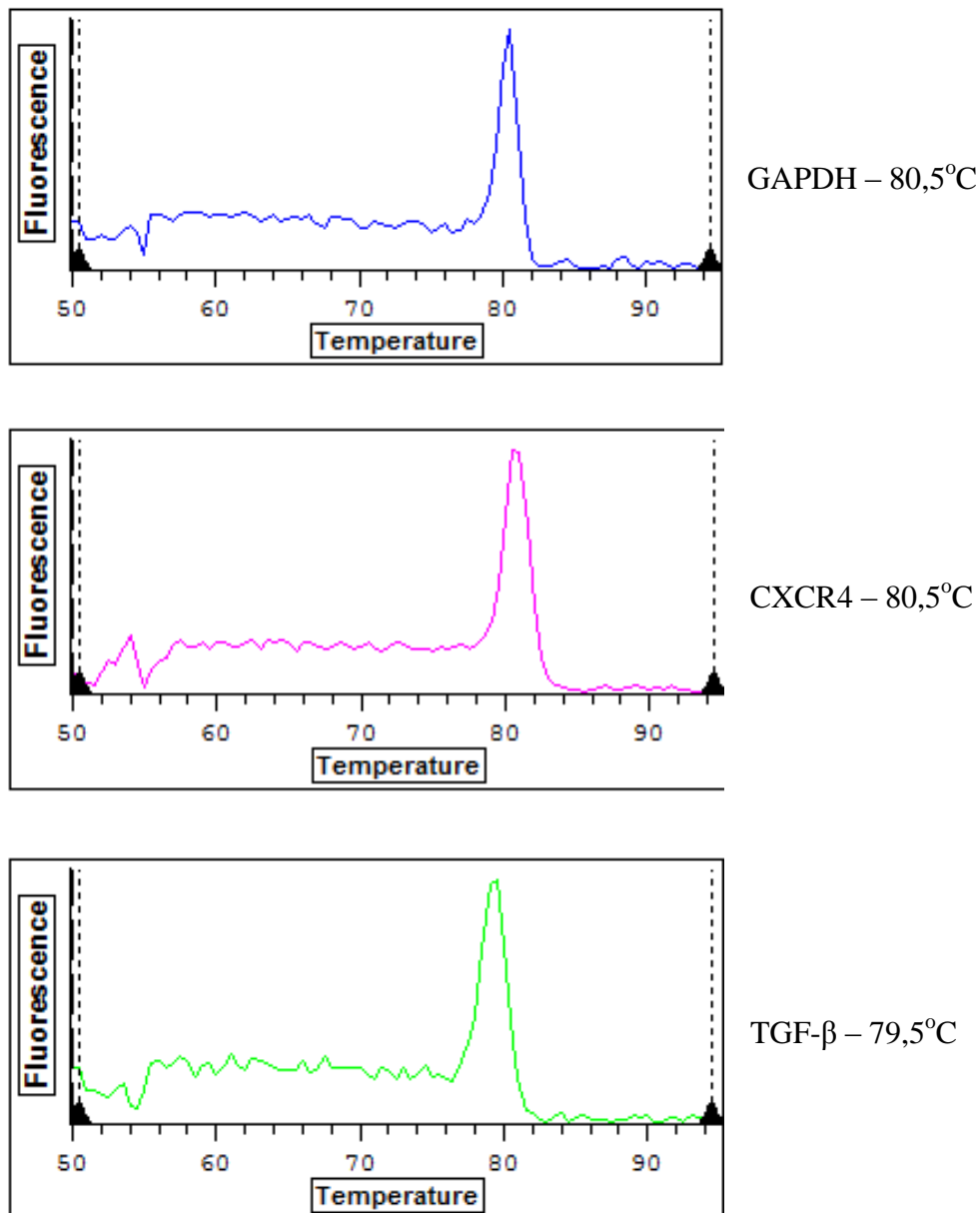


Figura 7 Perfil da Curva de *melting* dos genes GAPDH, CXCR4 e TGF- β realizada após a amplificação dos genes por RT-PCR quantitativo. Reação de RT-PCR quantitativo, utilizando o fluoróforo SYBR Green. Verificou-se a presença de picos nas temperaturas de melting dos genes, GAPDH – 80.5°C, CXCR4 – 80.5°C e TGF- β a 79.5°C.

Em relação à análise da expressão gênica do TGF- β , não houve diferença significativa quando realizada análise multivariada ajustada para as características clinicopatológicas, como faixa etária ($p=0,227$), expressão de receptor de estrógeno ($p=0,872$), expressão de receptor de progesterona ($p=0,872$), classificação histológica ($p=0,186$) e estadiamento do tumor ($p=0,216$), como demonstrado nas Figuras 8-10.

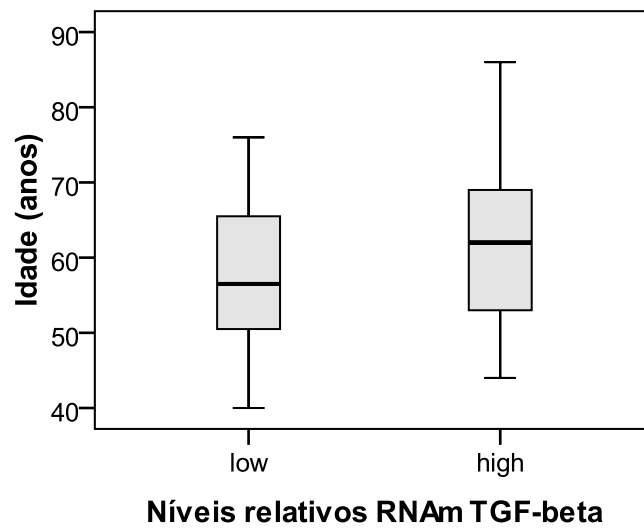


Figura 8 Expressão de RNAm TGF- β no tecido mamário de acordo com a idade. Não foi observado diferença significativa entre as variáveis pelo teste de *Kruskal-Wallis* ($p= 0,227$). Low representa as expressões negativas e High as expressões positivas.

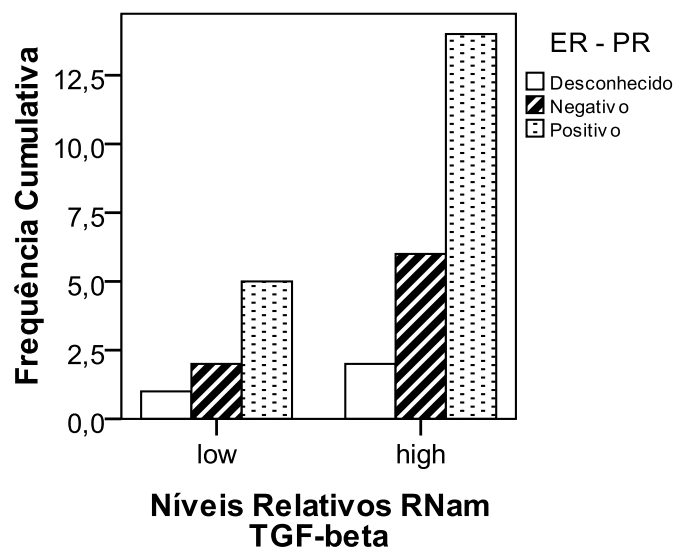


Figura 9 Expressão de RNAm TGF- β no tecido mamário em relação aos receptores de estrógeno e progesterona. Não foi observado diferença significativa entre as variáveis pelo teste de *Kruskal-Wallis* ($p= 0,872$). Low representa as expressões negativas e High as expressões positivas. (ER= Receptor de Estrógeno e PR= Receptor de Progesterona).

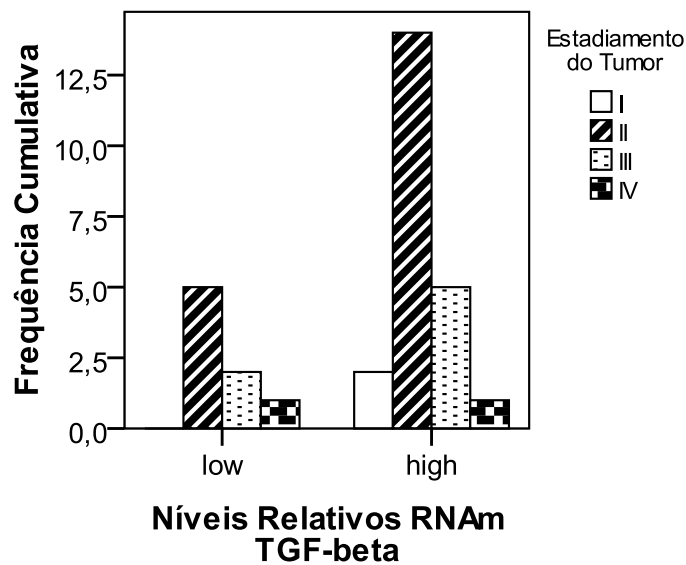


Figura 10 Expressão de RNAm TGF- β no tecido mamário em relação ao estadiamento do tumor. Não foi observado diferença significativa entre as variáveis pelo teste de *Kruskal-Wallis* ($p=0,216$). Low representa as expressões negativas e High as expressões positivas.

Entretanto, verificou-se associação entre menor expressão de TGF- β com maior tamanho de tumor ($p=0,025$) $\rho(\text{rho})=0,484$ (Figura 11) e associação entre menor expressão de TGF- β com maior número de linfonodos comprometidos ($p=0,033$) $\rho(\text{rho})=0,400$. (Figura 12).

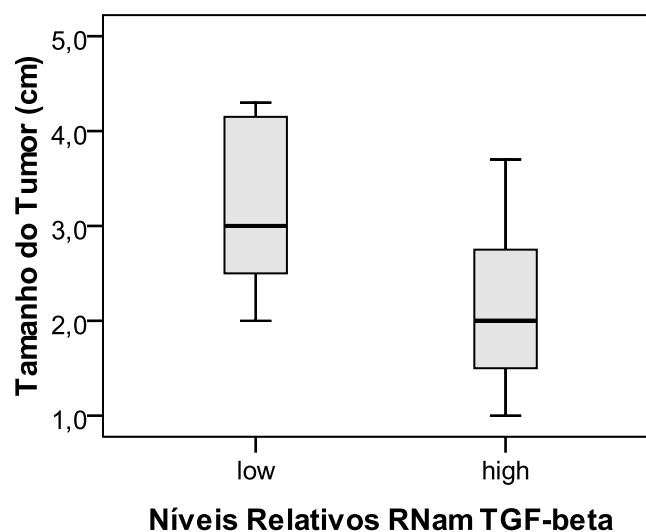


Figura 11 Expressão de RNAm TGF- β no tecido mamário em relação ao tamanho do tumor. Houve associação significativa entre menor expressão de TGF- β com maior tamanho de tumor pelo teste de Correlação de Pearson uni-caudado ($p=0,025$) $\rho(\text{rho})=0,484$.

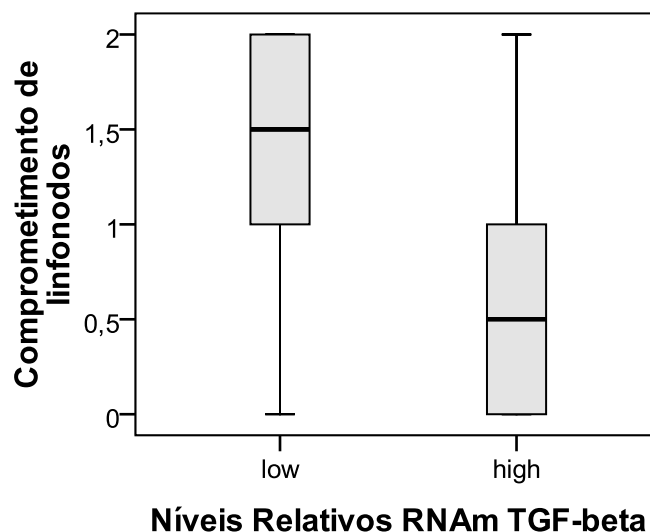


Figura 12 Expressão de RNAm TGF- β no tecido mamário em relação ao comprometimento de linfonodos. Houve diferença significativa entre menor expressão de TGF- β com maior comprometimento de linfonodos pelo teste de Pearson uni-caudado ($p=0,033$) $\rho(\text{rho}= 0,400)$.

O RNAm do TGF- β foi também analisado em relação às variantes alélicas polimórficas, e os pacientes com genótipo CC apresentaram uma expressão aumentada de TGF- β , apesar de não ter sido significativa em relação aos outros genótipos ($p=0,064$), como demonstrado na Figura 13.

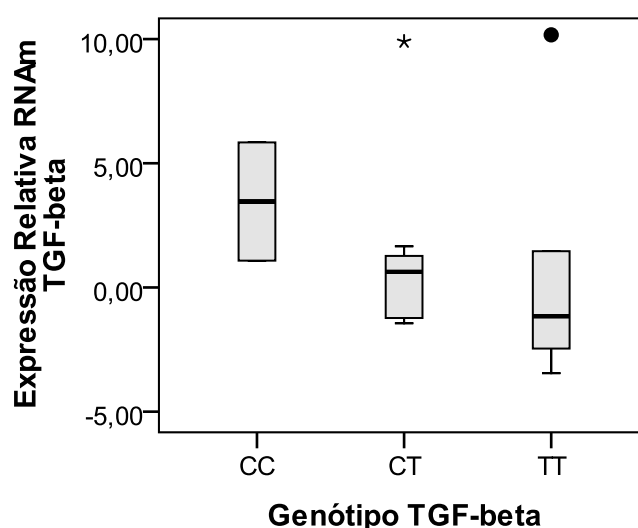


Figura 13 Expressão de RNAm TGF- β no tecido mamário de acordo com o genótipo de TGF- β . Não foi observado associação significativa entre as variáveis pelo teste de *Kruskal-Wallis* ($p= 0,064$). CC, CT e TT representam os genótipos do polimorfismo de TGF- β T869C. (* e • representam pontos discrepantes).

Na avaliação da expressão de RNAm de CXCR4 em relação aos parâmetros clinicopatológicos, nenhuma diferença estatisticamente significativa foi observada quanto a

idade da paciente ($p=0,939$), positividade para receptores hormonais ($p=0,112$), grau histológico ($p=0,646$), status nodal ($p=0,791$) e estadiamento tumoral ($p=0,192$).

Entretanto, quando comparada a expressão relativa de RNAm de TGF- β com CXCR4, foi observado correlação entre eles ($p= 0,020$) pelo teste de Kruskal-Wallis, como visto na Figura 14.

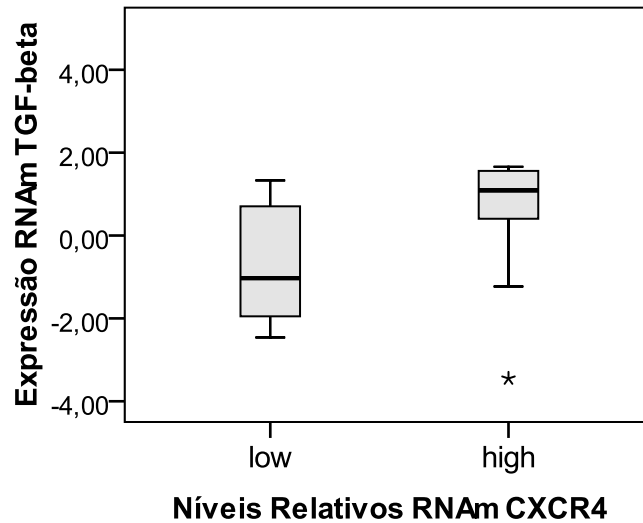


Figura 14 Expressão gênica de RNAm para CXCR4 e TGF- β . RNAm para CXCR4 e TGF- β foram calculados segundo valores de CT e eficiência (Pfaffl, 2001). A correlação foi avaliada pelo teste estatístico de Kruskal-Wallis. Low representa as expressões negativas e High as expressões positivas. (* representa ponto discrepante).

5 DISCUSSÃO

Atualmente os conhecimentos sobre os fatores de risco associados ao câncer são amplos, e estão em constante crescimento. Contudo, apesar dos avanços, a incidência do câncer de mama continua aumentando, ocupando o segundo lugar dentre os tipos de câncer mais freqüentes em todo o mundo, sendo o mais freqüente entre as mulheres (Brasil, 2009).

Parâmetros clinicopatológicos têm sido validados e servem como parâmetro para a terapia sistêmica e o prognóstico. Estão inclusos o tamanho do tumor, comprometimento dos linfonodos, grau histológico, tipo histológico, idade dos pacientes (Lacroix et al. 2004), perfil molecular e resposta a terapia (Rakha et al. 2009).

A incidência e a prevalência da maioria dos tumores aumentam com o avanço da idade (Fulop et al. 2010). A associação entre câncer e idade pode ser explicada por uma maior exposição a agentes carcinógenos em pessoas de idade mais avançada, o que levaria a uma disfunção tecidual causada pelo acúmulo de lesões celulares e moleculares (Malaguarnera et al., 2010; Hasty et al., 2003), uma vez que o envelhecimento está associado à incapacidade de manter e reparar células da linhagem somática (Kirkwood, 2000). A faixa etária das pacientes com câncer de mama envolvidas neste estudo variou de 40 a 76 anos, sendo que a idade mediana encontrada foi de 60 anos, o que está de acordo com os dados observados por Moura-Gallo et al. (2004), Moraes et al. (2006) e por Marinho et al. (2008), os quais relataram idades medianas de 57, 54 e 55,9 anos, respectivamente.

Estrógeno é um fator de crescimento que estimula a proliferação celular. Os efeitos do estrógeno são mediados através de receptores de estrógeno (ER). Por outro lado, TGF- β atua como inibidor do crescimento celular, e sua sinalização é mediada por Smads. Ito e colaboradores (2010) observaram que o estrógeno inibe a sinalização do TGF- β por degradar a proteína Smad. Embora inúmeros estudos têm demonstrado ligação entre a sinalização estrógeno/ER e TGF- β /Smad, mecanismos moleculares ainda precisam ser determinados (Ito et al, 2010).

Alguns estudos apontam que a agressividade dos tumores se deve ao fato de estarem relacionados ao receptor de estrógeno (ER) positivo ou negativo (INCA/MS, 2010). É conhecido que aproximadamente 70% dos cânceres de mama expressam receptor de estrógeno α (ER) e são considerados hormônio dependente (Ciocca e Elledge, 2000). De

acordo com estes dados, nosso estudo apresentou 61,9% de pacientes com câncer de mama que expressavam receptores de estrógeno e progesterona.

Em relação à classificação histológica, o presente estudo apresentou 95,24% das pacientes com carcinoma ductal invasivo (CDI), o que está de acordo com dados obtidos por Harris e Solin (2000), que verificaram incidência de 47 a 79% de CDI e 2 a 15% de carcinoma lobular invasor (CLI) em pacientes com tumores de mama.

Apesar de nosso estudo ter encontrado maior frequência de estadiamento II (61,9%) e III (23,81%), a maioria dos casos de câncer de mama no Brasil é diagnosticada em estágios avançados (III e IV), diminuindo as chances de sobrevivência das pacientes e comprometendo os resultados do tratamento (INCA/MS, 2007). Uma das causas no retardo do diagnóstico pode ser o reflexo da inexistência de uma política consistente de controle da doença através do diagnóstico precoce, que tem na mamografia o seu instrumento fundamental. Segundo Koch *et al* (2000), além de insuficientes em número, os mamógrafos existentes no Brasil encontram-se mal distribuídos, estando a sua grande maioria (76,7%) instalados em clínicas radiológicas privadas e com maior concentração nas regiões sudeste e sul do país.

O tamanho do tumor está diretamente relacionado ao risco de recidiva. Os tumores de menor tamanho estão invariavelmente relacionados a um melhor prognóstico tanto para sobrevivência global quanto para sobrevivência livre de doença, independente do autor, período ou tipo de tratamento aplicado (Abreu e Koifman, 2002). Quanto maior o seu tamanho, maiores são as chances da existência de comprometimento metastático dos linfonodos loco-regionais (Farley e Flannery, 1989; Palmer *et al*, 1982; Valagussa *et al*, 1978). Nosso estudo verificou que 55,56% das pacientes apresentaram tumor entre 2.1 a 5.0 cm.

A proliferação de células epiteliais de mama malignas é regulada por vários estímulos, incluindo citocinas e fatores de crescimento, assim as variações desses genes podem modificar o risco de câncer de mama individual (Lee *et al*, 2005). Resultados recentes têm revelado que o ganho e a perda de sinalização de TGF- β em carcinoma celular pode promover metástase (Bierie e Moses, 2009). Polimorfismos neste gene podem afetar os níveis de expressão desta citocina e assim alterar a susceptibilidade individual para cânceres, incluindo o de mama (Tang *et al*, 1998). Foi descrito que o polimorfismo T869C induz a um aumento sérico dos níveis de TGF- β 1 (Yokota *et al*, 2000; Ziv *et al*, 2001) e está relacionado a uma diminuição no risco de câncer de mama (Ziv *et al*, 2001; Kaklamani *et al*, 2005; Dunning *et*

al, 2003; Jin et al, 2004; Krippel et al, 2003; Le Marchand et al, 2004; Lee et al, 2005; Shin et al, 2005; Hishida et al, 2003).

No entanto, estudos epidemiológicos não encontraram associação significativa entre o risco de câncer de mama (Krippel et al., 2003; Le Marchand et al., 2004; Jin et al., 2004). Hishida et al (2003) relataram que o genótipo CC estava intimamente relacionado com menor risco de câncer de mama quando comparado com o genótipo TT em mulheres japonesas. Este trabalho verificou uma prevalência de genótipo heterozigoto (TC; Leu/Pro) (61.91%), enquanto o genótipo TT (Leu) foi observado em 28.57% e o genótipo homozigoto CC (Pro) foi observado somente em duas pacientes (9.52%). Em razão ao pequeno número de amostra neste trabalho, não foi realizado um estudo de associação caso-controle, mas apenas a comparação do polimorfismo e a expressão de TGF- β com os dados clinicopatológicos.

Não foi observado associação entre o polimorfismo TGF- β T869C com as características clinicopatológicas (Tabela 1), mas houve uma tendência de associação entre o genótipo homozigoto TT com receptores hormonais (Figura 5).

Pacientes homozigotos para prolina têm aumento plasmático de TGF- β (Grainger et al, 1999; Yokota et al, 2000). Este resultado é compatível com nosso estudo, pois houve um aumento de expressão deste gene em pacientes homozigotos CC quando comparado a expressão de tecido mamário (Figura 13).

Bierie e Moses (2009) demonstraram que a perda de sinalização pelo TGF- β no interior de células do carcinoma pode suprimir a expressão de quimiocinas que facilitam a interação das células tumorais mielóides na promoção de metástase. Os resultados de Zhao et al (2010) sugerem uma ligação entre a expressão de TGF- β e CXCR4 em células humanas de câncer de mama MCF-7, que poderia ser um dos mecanismos pelo qual o TGF- β medeia o aumento do potencial metastático das células cancerosas de mama. Apesar de não ter sido encontrado diferença significativa entre a expressão de CXCR4 e os dados clinicopatológicos, nosso estudo observou pela primeira vez uma associação entre a expressão de TGF- β e CXCR4 em tecido mamário ($p=0,020$) (Figura 14).

É conhecido que a superexpressão de TGF- β no tecido tumoral e no estroma pode facilitar o desenvolvimento de metástases, principalmente pelo TGF- β estimular angiogênese e aumentar a mobilidade da célula tumoral (Dumont e Arteaga, 2000). Quando avaliamos a expressão gênica do TGF- β com as características clinicopatológicas, não observamos

diferença significativa em relação à idade ($p=0,227$), receptores hormonais ($p=0,872$), classificação histológica ($p=0,186$) e estadiamento do tumor ($p=0,216$), o que está de acordo com o trabalho de Delvenne et al (1992) que também não verificaram correlação entre expressão de TGF- β com parâmetros clínicos em tumor de mama.

Entretanto, houve associação significativa entre baixas expressões de TGF- β e aumento do tumor ($p=0,025$) $\rho(\text{rho}= 0,484)$ (Figura 11), assim como baixas expressões de TGF- β e maior comprometimento linfático ($p=0,033$) $\rho(\text{rho}= 0,400)$ (Figura 12). O risco dos linfonodos axilares estarem comprometidos é diretamente proporcional ao tamanho do tumor. Os tumores até 1 cm de diâmetro apresentam a probabilidade média de 20% a 30% de estarem envolvidos pela doença, sendo que os tumores ductais com grau histológico elevado podem até dobrar o percentual de comprometimento dos linfonodos (Carter et al, 1989; Fentiman et al, 1996).

De acordo com a literatura, o TGF- β tem mostrado suprimir a atividade anti-tumoral de células T, células NK, neutrófilos, monócitos e macrófagos que são conhecidos por ter um papel significativo na regulação da progressão do tumor (Li et al., 2006; Wrzesinski et al., 2007). Carcinomas geralmente secretam excesso de TGF- β e respondem a este excesso com invasão e metástase. Abordagens terapêuticas objetivam inibir este fenótipo invasivo induzido pelo TGF- β , mas também tentar manter seus efeitos de induzir a apoptose e inibir o crescimento (Akhurst e Rik Derynck, 2001).

Murray et al (1993) encontraram que pacientes com altos níveis de expressão de RNAm de TGF- β apresentam um grande período livre da doença. A aparente oposição desta relação entre TGF- β e progressão da doença sugere que este gene pode ter diferentes efeitos na progressão do câncer de mama dependendo do estágio da doença (Mu et al, 2008).

A via do TGF- β regula a iniciação, progressão e metástase tumoral. Nos estágios iniciais o TGF- β apresenta habilidade supressora da tumorigênese através da inibição da progressão do ciclo celular e indução de apoptose. O impacto citostático e apoptótico do TGF- β como supressor de tumor foi equilibrado por observações claramente demonstradas que o TGF- β poderia promover a progressão tumoral através da supressão da vigilância imunológica (Bierie e Moses, 2009).

No entanto, já foi demonstrado que a perda de capacidade de resposta das células do carcinoma à estimulação de TGF- β também pode promover metástase. Curiosamente, a

metástase avançada na ausência de uma resposta das células do carcinoma ao estímulo de TGF- β tem sido demonstrado que há um aumento da produção de quimiocinas, resultando no recrutamento de populações celulares pro-metastáticas mielóides supressoras para o microambiente do tumor (Bierie e Moses, 2010).

Assim, as observações sugerem que ele funciona como um supressor ou promotor tumoral, dependendo do contexto de estimulação. Embora o impacto do TGF- β na célula de carcinoma seja significativo, é geralmente aceito que a progressão primária da metástase do carcinoma é regulada por uma complexa rede de interações entre células do tumor e do hospedeiro. No entanto, muitos dos estudos iniciais não foram capazes de controlar as influências locais e sistêmicas da expressão de TGF- β exógeno no microambiente do tumor mamário (Bierie et al., 2008).

Linfócitos, incluindo células T, Tregs e *natural killer* (NK), e os padrões de citocinas liberados estão envolvidos na prevenção do câncer de mama primário e recorrência. O prognóstico do câncer pode estar relacionado ao estado funcional do sistema imunológico (Standish et al. 2008). Durante a última década, reflexões têm sido adquiridas em relação aos mecanismos subjacentes à interação dinâmica entre células do sistema imunológico e a progressão do tumor. Os dados acumulados indicam que o resultado de uma resposta imune na direção de um tumor é amplamente determinado pelo tipo de resposta imune elicitada.

Vários estudos têm indicado uma correlação positiva da expressão do fator de crescimento do endotélio vascular (VEGF - *Vascular endothelial growth factor*) com a vascularização e malignidade do tumor (Singha et al, 2010). Células T regulatórias (Tregs) ativadas liberam altos níveis de TGF- β e tem sido sugerido, que indiretamente induzem a expressão do VEGF e leva ao aumento da vascularização e progressão tumoral (Giatromanolaki et al, 2008; Gupta et al, 2007).

Estudos demonstraram que as Tregs também podem ser geradas a partir de células T derivadas do tumor (Berger et al, 2005; Karube et al, 2004) e a expressão de RNAm de Foxp3, IL-10, TGF- β 1 e CCL22 são significativamente altos no tecido canceroso quando comparados ao tecido normal. Além de responder ao estímulo do TGF- β , as Tregs também produzem esta citocina (Akhurst e Derynck, 2001; Kalluri e Zeisberg, 2006; Pollard, 2004; Wels et al, 2008). Recentemente, foi sugerido que as Tregs estão envolvidas no início e na progressão do câncer de mama primário humano, possivelmente contribuindo a um pior prognóstico (Ohara et al, 2009).

Embora seja comumente aceito que o comportamento clínico dos tumores depende da interação entre as células tumorais e as células do hospedeiro, muitas pesquisas moleculares tem identificado marcadores derivados dos tumores, enquanto pouco é descrito sobre os fatores dos hospedeiros e de seus promissores papeis na patogênese tumoral.

Especula-se que mais investigações com informações completas sobre potenciais fatores de contradição e abrangência de dados de genotipagem precisam ser conduzidos para concluir-se sobre o papel do TGF- β no desenvolvimento do câncer de mama. A invasão, o tamanho e a vascularização são parâmetros de prognóstico no câncer de mama e a constatação de uma correlação positiva entre o TGF- β e expressão de CXCR4, uma correlação inversa com o tamanho do tumor na expressão de TGF- β e uma associação entre baixas expressões de TGF- β com maior comprometimento de linfonodos por si só, sugere um papel desses genes como fatores emergentes no microambiente do câncer de mama.

6 CONCLUSÃO

- O genótipo do polimorfismo TGF- β T869C CC foi observado em 9,52% (02/21), o genótipo CT em 61,91% (13/21) e o genótipo TT em 28,57% (06/21) das pacientes. Não foi observado diferença significativa na distribuição genotípica do TGF- β T869C de acordo com as características clinicopatológicas, como faixa etária ($p=0,224$), a expressão de receptor de estrógeno ($p=0,092$), expressão de receptor de progesterona ($p=0,092$), classificação histológica ($p=0,287$), tamanho do tumor ($p=0,423$) e estadiamento do tumor ($p=0,479$).
- Também não foi observada diferença significativa entre a frequência alélica do TGF- β com as características clinicopatológicas das pacientes, como faixa etária ($p=0,157$), a expressão de receptores hormonais ($p=0,495$), classificação histológica ($p=0,490$), tamanho do tumor ($p=0,495$) e estadiamento ($p=0,317$).
- Os pacientes homozigotos CC para o polimorfismo TGF- β T869C apresentaram uma maior expressão de TGF- β , embora não foi estatisticamente significante ($p= 0,064$).
- Apesar de não ter sido encontrado correlação da expressão de CXCR4 com os parâmetros clinicopatológicos, houve correlação de expressão relativa de TGF- β com CXCR4 ($p= 0,020$).
- Não houve diferença significativa da expressão gênica do TGF- β quando comparada com as características clinicopatológicas, como faixa etária ($p=0,227$), expressão de receptor de estrógeno ($p=0,872$), expressão de receptor de progesterona ($p=0,872$), classificação histológica ($p=0,186$) e estadiamento do tumor ($p=0,216$). Entretanto, foi observado associação entre menor expressão de TGF- β com maior tamanho de tumor ($p=0,025$) $\rho(\text{rho}= 0,484)$ e menor expressão de TGF- β com maior comprometimento de linfonodos ($p=0,033$) $\rho(\text{rho}= 0,400)$.

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Anexos

Emerging factor in breast cancer microenvironment: TGF- β T869C polymorphism and its expression associated with CXCR4 expression

ABSTRACT

The transforming growth factor beta (TGF- β) is a cytokine that appears to exert two major functions, tissue homeostasis and the response to tissue injury. The immunopathology associated with hyperactivation of the TGF- β pathway in tumor progression is one of the main reasons that have attracted attention for this gene as a novel therapeutic target. CXCR4, a seven transmembrane G-coupled receptor protein is expressed on a wide variety of tissue and organ specific stem cells and it is known that is overexpressed in cancer and plays a role in invasion and metastasis. The propose of this study was to investigate the TGF- β T869C polymorphism and its expression correlated with CXCR4 expression in breast cancer patients. DNA and RNA from 21 patients were analyzed for the TGF- β polymorphism and TGF- β and CXCR4 expression by qRT-PCR. Regarding the allele frequency it was observed that the allele C obtained a distribution of 0.43 and a T allele distribution of 0.56 and there was no significant difference in genotype distribution according to clinic pathological characteristics. The homozygous CC patients presented a higher TGF- β expression, although not significant. The relative mRNA expressions of CXCR4 and TGF- β were compared and a positive correlation was observed ($p= 0.020$). There was also an association between lower expression of TGF- β with increasing tumor size ($p = 0.025$) and with lymph node involvement ($p = 0.033$). Our findings, including the positive correlation between TGF- β and CXCR4 expression and the correlation of tumor size as the lymph node involvement with low expression of TGF- β , suggests a role as a progression markers for breast carcinoma.

Keywords: breast cancer, TGF- β , CXCR4, qRT-PCR

INTRODUCTION

The transforming growth factor beta (TGF- β) family of polypeptides comprises a group of highly conserved dimeric proteins with a molecular weight of approximately 25 kDa (Roberts et al., 1993). They are ubiquitously expressed in eukaryotes and typically secreted into the extracellular milieu in an inactive form, where they become locally activated in response to the appropriate stimuli (Wakefield et al., 1987; Annes et al., 2003).

It is known that in self-renewing epithelia, which are the most common sites of origin of cancer, TGF- β appears to exert two major functions, tissue homeostasis and the response to tissue injury (Tan et al., 2009). TGF- β signaling plays a key role in maintaining vascular integrity (Maharaj et al., 2008; Stuhmann et al., 2007; Goumans et al., 2003). This process probably involves a basal level of “endogenous” TGF- β signaling, which protects against the development of early neoplastic lesions.

The immunopathology associated with hyperactivation of the TGF- β pathway in tumor progression is one of the main reasons that have attracted attention for this gene as a novel therapeutic target (Li and Flavell, 2008; Wrzesinski et al., 2007).

The expression of TGF- β is under gene control. So far, TGF- β polymorphisms have been identified: three upstream of exon 1, an insertion/deletion of a cytosine residue within the 5' untranslated region, and three in the coding region of the gene, which result in amino acid substitutions and might therefore affect TGF- β structure and/or function (Awad *et al* 1998, Cambien *et al* 1996). 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). This transition disrupts the structure (Cambien *et al* 1996, Randall *et al* 1989) and results in increased levels of TGF- β protein (Dunning *et al* 2003, Grainger *et al* 1999, Suthanthiran *et al* 2000) and mRNA (Suthanthiran *et al* 2000) in individuals with the proline allele with a 2.8-fold increase in TGF- β secretion compared with the leucine allele in vitro (Kirshner et al 2006).

The TGF- β C-allele were shown to be associated with increased of TGF- β serum level and increased breast cancer risk among C allele carriers with invasive breast cancer (Dunning et al., 2003). Likewise, the tumor promoting effect of TGF- β could explain increased breast cancer risk for C allele carriers. According to the same authors, the effect of TGF- β 29C-allele containing genotypes (TC or CC) were stronger for invasive cases whose lymph nodes were infiltrated with cancer cells.

The results from Lee et al. (2005) suggest that TGF- β T29C and TNF- β A252G polymorphisms may modify individual susceptibility to breast cancer in postmenopausal Korean women. Although, further investigation with more comprehensive information of potential confounders and genotyping need to be conducted.

CXCR4, a seven transmembrana G-coupled receptor protein, originally identified as a coreceptor for T cell line tropic strains of human immunodeficiency virus, is expressed on a wide variety of tissue and organ specific stem cells (Kucia et al., 2005).

It is known that CXCR4 is overexpressed in cancer and plays a role in invasion and metastasis (Balkwill, 2002, Muller et al, 2001; do Val Carneiro et al, 2009) and together with its ligand CXCL12 are suggested to be involved in migration, invasion and metastasis of breast cancer cells (Muller et al., 2001; Kang et al., 2003; Smith et al., 2004; Liang et al., 2005; Holland et al., 2006). Kang et al. (2005) has been demonstrated through CXCR4 transcript and protein levels in human breast cancer tissues, that the level of CXCR4 expression is significantly correlated with lymph node metastasis and suggest that this receptor may be a useful prognostic indicator and a potential therapeutic target in cancer therapies for breast cancer.

The results from Zhao et al (2010) suggest a link between TGF- β and CXCR4 expression in MCF-7 human breast cancer cells, which may be one of the mechanisms of TGF- β mediated enhancement of metastatic potential in breast cancer cells. The propose of this study was to investigate the TGF- β T869C polymorphism and also its expression correlated with CXCR4 expression in breast cancer patients.

MATERIALS AND METHODS

Human Subjects and samples

The protocol was approved by the institutional Human Research Ethics Committee of the State University of Londrina, Paraná, Brazil. The patients were invited to participate, informed in detail regarding the research and voluntary written consent term was obtained from all of the patients enrolled. A term of free informed consent was signed by all sample donors and doctors involved prior to blood or tissue collection.

Samples of invasive breast carcinoma tissue and normal mammary gland tissue were obtained from a case series of 21 patients who had undergone surgery at the Londrina's Cancer Institute, Parana State, Brazil. Clinical staging was determined according to the Union of International Control of Cancer (UICC) classification criteria. Normal breast tissue was largely from the contralateral tumor of patients undergoing surgery for the tumors. 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 peripheral blood leukocytes of patients (Kirby et al., 1990) or from blocks of breast tissue previously fixed in formalin and embedded in paraffin for polymorphisms analyses (Isola et al, 1994). Briefly, DNA was extracted in the presence of 0.2M NaCl and 0.25% SDS, for 4h at 37°C. After precipitation with ethanol, the pellet was dried and resuspended in 50µL of *milli Q* water. All samples of DNA extracted were analyzed for integrity on agarose gel 1% (w/v). All samples showed single bands indicating the integrity of DNA.

Polymerase Chain Reaction (PCR): TGF-β

DNA (200 ng) was amplified by polymerase chain reaction (PCR) with specific primers for TGF-β polymorphism (T869C) in according for Lee et al modified (2005), following the GenBank accession number NG_013364.1. TGF-β (T) forward 5'- GGG CTG CGG CTG CTG CT -3'; TGF-β (T) reverse 5'- GTA GTC GGC CTC AGG CTC GG -3'; TGF-β (C) forward 5'- CTC CAC CAC TGC GCC CTT CT -3' and TGF-β (C) reverse 5'- AGC AGC GGT AGC AGC AGC G -3'. Samples were amplified using the kit buffer plus 1.25 units of Taq polymerase (InvitrogenTM, Carlsbad, California, USA). PCR conditions were: 10 min denaturation at 95°C, 35 cycles of 1 min at 94°C, 1 min at 69°C (for allele T) or 65°C (for allele C) and 1 min at 72°C, and 5 min elongation at 72°C in a thermocycler (PCR-Sprint Hybaid - Guelph, Ontario, Canada). Amplicons of 297 base pairs for TGF-β (T) and 204 base pairs for TGF-β (C) were analyzed by electrophoresis on acrylamide gel (10%) and detected by a nonradioisotopic technique using a commercially available silver staining method.

RNA isolation and reverse transcriptase reaction

Total cellular RNA was extracted from 21 samples of breast cancer tumor and healthy mammary tissue without any apparent histological abnormality with TRIzol LS reagent (InvitrogenTM, Carlsbad, California, USA) according to the manufacturer's instructions. Purified total RNA was measured and assessed for purity by determining absorbance at 260 and 280 nm and then was stored at -20°C until testing. Reverse transcriptase reaction was performed using 500ng of RNA according to Carneiro et al., 2009. Prior to the tests with CXCR4 and TGF-β mRNA, it was performed a validation of human RNA samples and cDNA quality by RT-PCR for the gene of β-actin. PCR for beta-actin cDNA was determined as described by Amarante et al. (2005) and PCR conditions were: 94°C for 1 min followed by 35

cycles of 94°C for 30 sec, 55°C for 30 sec, 72° C for 1 min and finally, 72°C for 10 min in a Hybaid PCR Sprint Thermal Cycler (Biosystems, Guelph, Ontario, Canada). All RNA samples had detectable amounts of mRNA for β -actin and showed no degradation. There was no contamination by genomic DNA, once all the amplified products showed a single fragment corresponding to 353 bp.

Quantitative real-time PCR for CXCR4 mRNA and TGF- β mRNA

The human glyceraldehyde 3-phosphate dehydrogenase gene (GAPDH) was used for quantitative PCR in the analysis of gene expression in human cells. Besides the values of CT used for determining the total amount of cDNA used were considered the best efficiency values of the Optical Monitor software (BIO-RAD). From these tests, we used the amount of 20 ng of cDNA for quantitative PCR for TGF- β genes, CXCR4 and GAPDH. The sequences of primers for TGF- β and CXCR4 were obtained according to the analysis in GeneRunner Software (Hasting Software Inc., New York, USA) (Table 1).

Real-time PCR using SYBR green fluorescence was performed with 20 ng of cDNA in a total volume of 20 μ L. Quantitative real-time PCR reaction was carried out using Platinum[®] SYBR Green qPCR SuperMix UDG (Invitrogen[™]) with 0.25 μ M of each sense and antisense primers (described below). The PCR reaction was performed for 40 cycles as follows: 95°C for 30 sec, 54°C for 30 sec and 72°C for 30 sec in a Chromo4[™] Real Time PCR Detection (Bio-Rad, Hercules, USA). A melting curve analysis was consistently performed at the end of the reaction to check for primer-dimer artifacts and contamination. In addition, in all experiments, appropriate negative controls containing no template were subjected to the same procedure to exclude or detect any possible contamination.

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

Gene	GenBank Accession Number	Primer	Sequence	Melting Temperature (T°C)
CXCR4 mRNA	AF025375	<i>Foward</i>	5' TCTACTCCATCATCTTCTTTA 3'	80.5
		<i>Reverse</i>	5' ACGTTGGCAAAGATGAAGGTC 3'	
TGF β mRNA	NM_000660	<i>Foward</i>	5' GTC GGG AGA AGA GGA AAA AAA 3'	79.5
		<i>Reverse</i>	5' GGC AAA GGG AGG CCG TC 3'	
GAPDH mRNA	NM_002046	<i>Foward</i>	5' GAAGGTGAAGGTCGGA 3'	80.5
		<i>Reverse</i>	5' GGGTCATTGATGGCAAC 3'	

Statistical Analysis

Statistical analysis was performed using SPSS Statistics 17.0 (SPSS inc. Chicago, Illinois, USA). For analysis of genotype-related gene expression and clinicopathological variables, we used the Kruskal Wallis. In quantitative RT-PCR analysis, the expression level of CXCR4 and TGF- β mRNA was calculated according to the Pfaffl method (Pfaffl, 2001), in which C(t) values for target gene were the mean fold change + SEM for three independent determinations corrected by GAPDH gene C(t) values from control samples, considering efficiency values. To analyze the relative expression of mRNA was used the Kruskal-Wallis and to determine the correlation between the expression of TGF- β with the clinicopathological characteristics, we used the Pearson correlation test. A *p* value <0.05 was considered statistically significant.

RESULTS

Clinicopathological features

The age of patients involved in this study ranged from 40 to 76 years, with the highest incidence of breast cancer observed in women with age over than 60 years (42.86%, 09/21). Most patients diagnosed with breast cancer showed invasive ductal carcinoma (95.24%, 20/21), according to clinical criteria established by the Union of International Control of Cancer (UICC, 2002) and among them, the tumors staging II and III (85.71%, 18/21) were the most prevalent, while only 14.28% (03/21) had tumors of stage I or IV. Tumor size predominant among the patients ranged from 2.1 - 5.0 cm, which corresponds to approximately 57.10% (12/21). The presence of estrogen and progesterone receptors (61.90% - 13/21) was observed in the same patients (Table 2).

TGF- β T869C polymorphism analysis

The TGF- β T869C polymorphism was assessed by PCR from DNA samples of peripheral blood or block of formalin-fixed, paraffin-embedded. Analysis of electrophoresis of amplified fragments allowed the visualization of three different genotypes: TT, CC and TC. (Figure 1).

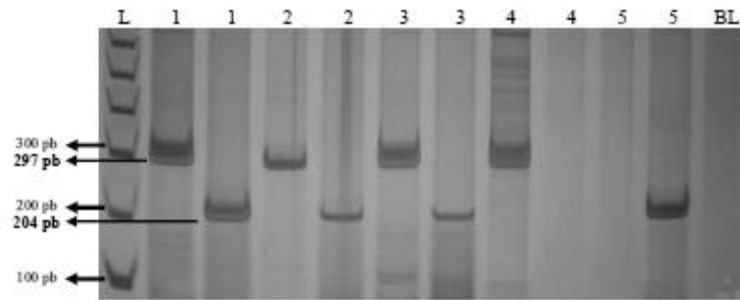


Figure 1. Electrophoretic profile of TGF- β polymorphism T869C. Polyacrylamide gel 10% (v / v) stained with silver nitrate. L – ladder 100 bp (Invitrogen), samples of 1-3 show amplification of 297bp for the T allele and 204bp for allele C and BL – blank reaction (negative control). Sample 1-5 show amplification of 297bp for the T allele and 204bp for allele C and BL corresponds to the blank (negative control). Samples 1-3 corresponds to TC genotype, sample 4 the TT genotype and sample 3 CC genotype.

The CC genotype was observed in 9.52% (02/21), the CT genotype in 61.91% (13/21) and TT genotype in 28.57% (06/21) (Figure 2). Regarding the allele frequency it was observed that the allele C obtained a distribution of 0.43 and a T allele distribution of 0.56.

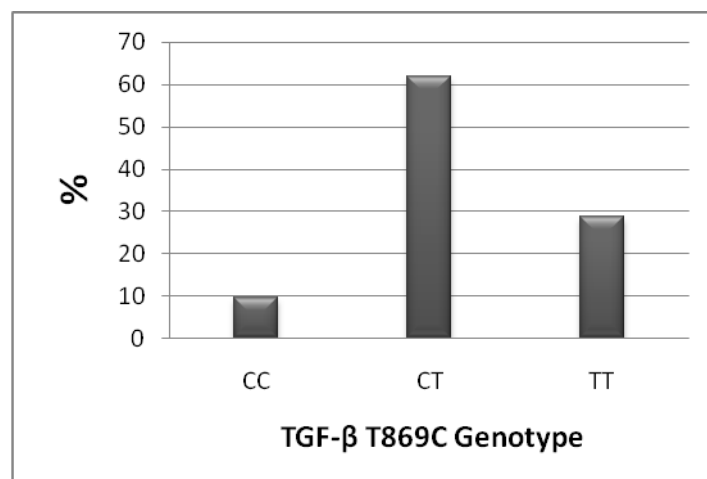


Figure 2. Genotype distribution of TGF- β polymorphism T869C. CC genotype was observed in 9.52% of patients, the CT genotype was found in 61.91%, while the TT genotype was observed in 28.57% of patients. ($X^2 = 1307$ for HWE, a degree of freedom, $p > 0.05$).

There was no significant difference in genotype distribution of TGF- β T869C according to clinicopathological characteristics evaluated, such as age ($p = 0.224$), the expression of estrogen receptor ($p = 0.092$), expression of progesterone receptor ($p = 0.0092$), histological classification ($p = 0.287$), tumor size ($p = 0.423$) and tumor stage ($p = 0.479$) as shown in Table 2.

Table 2. Clinicopathological characteristics of patients with breast cancer according to TGF- β T869C polymorphism.

		Number of individuals N (%) (n=21)	TGF- β Genotype		
			CC N(%)	CT N (%)	TT N (%)
Age (years)	< 40	1 (4.76)	0 (0.00)	0 (0.00)	1 (4.76)
	41 – 50	4 (19.05)	0 (0.00)	2 (9.52)	2 (9.52)
	51 – 60	7 (33.33)	1 (4.76)	4 (19.05)	2 (9.52)
	> 60	9 (42.86)	1 (4.76)	7 (33.33)	1 (4.76)
Estrogen (ER) and Progesterone (PR) Receptor	Positive	13 (61.90)	1 (4.76)	6 (28.57)	6 (28.57)
	Negative	6 (28.57)	1 (4.76)	5 (23.81)	0 (0.00)
	Unknow	2 (9.52)	0 (0.00)	2 (9.52)	0 (0.00)
Tumor Histology^a	IDC	20 (95.24)	2 (9.52)	13 (61.90)	5 (23.81)
	ILC	1 (4.76)	0 (0.00)	0 (0.00)	1 (4.76)
Tumor Size	0 – 2.0	8 (38.10)	0 (0.00)	7 (33.30)	1 (4.80)
	2.1 – 5.0	12 (57.10)	2 (9.50)	5 (23.80)	5 (23.80)
	> 5.1	1 (4.80)	0 (0.00)	1 (4.80)	0 (0.00)
Tumor Stage	I	2 (9.52)	0 (0.00)	1 (4.76)	1 (4.76)
	II	13 (61.90)	2 (9.52)	7 (33.33)	4 (19.05)
	III	5 (23.81)	0 (0.00)	4 (19.05)	1 (4.76)
	IV	1 (4.76)	0 (0.00)	1 (4.76)	0 (0.00)

^a IDC – Invasive Ductal Carcinoma; ILC – Invasive Lobular Carcinoma

All patients positive for estrogen receptor were also positive for progesterone receptor. Although not statistically significant, the Figure 3a shows a trend of association of the homozygous TT genotype with positive receptor for estrogen and progesterone, while Figure 3b, shows that the majority of patients who had increased size of tumor were estrogen receptor positive, or hormone dependent.

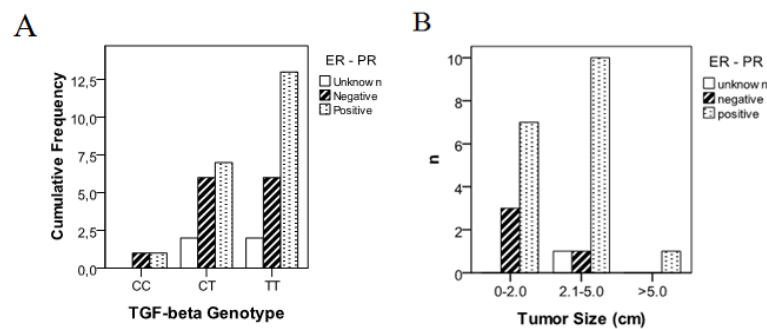


Figure 3. Assessment related to hormone receptor. A) genotypic distribution according to hormone receptors (estrogen and progesterone) of breast cancer patients. 28.57% of patients were ER + and PR + and belonged to genotype TT. (ER: Estrogen Receptor and PR = Progesterone Receptor). Kruskal-Wallis Test, $p = 0.092$. B) Distribution of hormone receptors according to tumor size. Kruskal-Wallis Test, $p = 0.727$.

Although the occurrence of the CT genotype was prevalent among patients with breast cancer, most patients with the homozygous TT genotype showed positivity for estrogen receptor and progesterone and the prevalence of tumor size was 2.1 - 5.0 cm (Figure 3).

There was a predominance of stage II between patients (61.90%), of which 33.33% had genotype CT and 19.05% had TT genotype, also the majority of patients with TT genotype had a tumor between 2.1 - 5.0 cm, the major size of tumor found in our sample, since only one patient demonstrated a tumor bigger than 5.0 cm.

Quantitative analysis of mRNA gene expression

Relative expression of mRNA of TGF- β and CXCR4 was assessed by RT-quantitative PCR in breast tissue. RNA was diluted and a curve was constructed to determine the total amount of cDNA to go to real time PCR, the choice was based on the average values of CT (Cycle Threshold) from duplicates. The calculations were carried out according Pfaffl, 2001, taking into account the efficiencies of the reactions.

Analysis of melting curve, revealed the presence of only one peak corresponding to the fragments of GAPDH (the temperature of 80.5 °C), CXCR4 (the temperature of 80.5 °C) and TGF- β (the temperature of 79.5 °C).

On the analysis of gene expression of TGF- β , no significant difference was verified when accomplished this multivariate analysis adjusting for clinicopathological characteristics such as age ($p = 0.227$), expression of estrogen receptor ($p = 0.872$), expression of receptor progesterone ($p = 0.872$), histological classification ($p = 0.186$) and tumor stage ($p = 0.216$). However, there was a positive association between lower expression of TGF- β with increased tumor size ($p = 0.025$) $\rho(\text{rho} = 0.484)$ (as shown in Figure 4) and there was association between lower expression of TGF- β with lymph node involvement ($p = 0.033$) $\rho(\text{rho} = 0.400)$ (Figure 5).

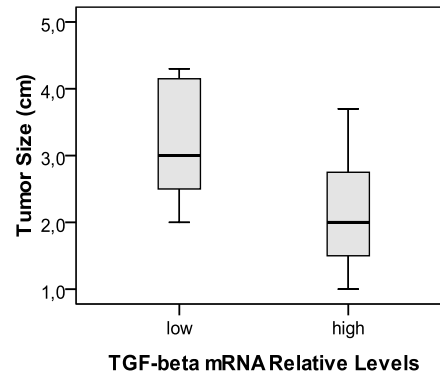


Figure 4. mRNA expression of TGF- β in mammary tissue in relation to tumor size. An inverse correlation between TGF- β mRNA levels and tumor size was observed by Pearson correlation test uni-caudate ($p = 0.025$) $\rho(\text{rho}) = 0.484$).

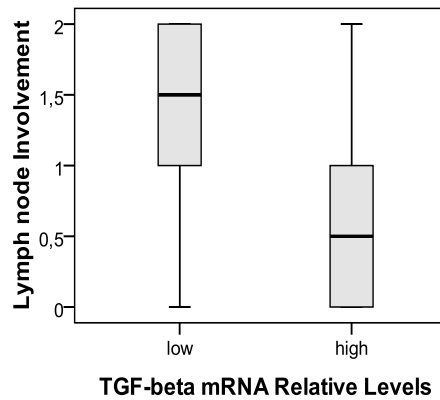


Figure 5. mRNA expression of TGF- β in mammary tissue in relation to lymph node involvement. Significant difference between lower expression of TGF- β with higher lymph node involvement by Pearson correlation test uni-caudate ($p = 0.033$) $\rho(\text{rho}) = 0.400$).

In the next step TGF- β mRNA expression was assessed according to T869C polymorphism, CC patients presented a higher TGF- β expression, although not significant when compared to other genotypes ($p=0.064$) (Figure 6).

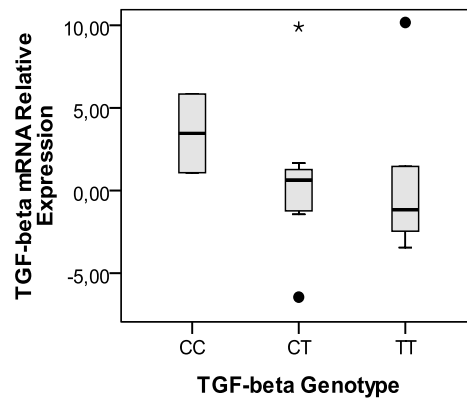


Figure 6. Expression of TGF- β mRNA in breast tissue according to the genotype of TGF- β . No significant difference was observed between the variables by Kruskal-Wallis test ($p = 0.064$). DC, CT and TT genotypes representing the TGF- β T869C polymorphism.

Regarding CXCR4 mRNA expression no significant difference was observed according to clinicopathological features analyzed such as patient age range ($p=0.939$), estrogen and progesterone receptor status ($p=0.112$), tumor histology ($p=0.646$), nodal status ($p=0.791$) and tumor stage ($p=0.192$).

However, when compared the relative expression of mRNA of TGF- β with relative expression of mRNA CXCR4, we observed a correlation between them ($p = 0.020$) by the Kruskal-Wallis Test, as seen in Figure 7.

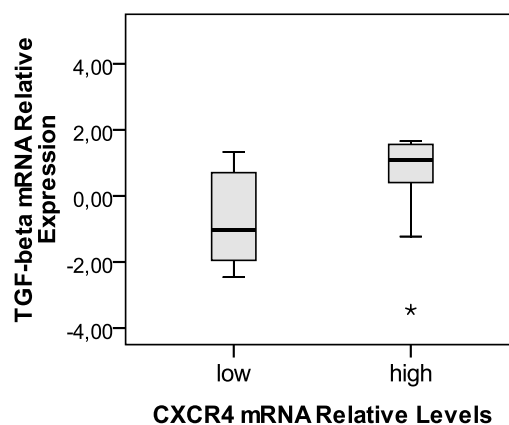


Figure 7. Gene expression of CXCR4 mRNA and TGF- β . CXCR4 mRNA and TGF- β were calculated according to values of TC and efficiency (Pfaffl, 2001). The correlation was evaluated by Kruskal-Wallis Test ($p=0.020$). Low represents the negative expressions and the high positive expressions. (* Represents discrepancy point).

DISCUSSION

Currently the knowledge about the risk factors associated with cancer is large, and are constantly growing. However, despite advances, the incidence of breast cancer continues to increase, ranking second among the most common cancers worldwide, being the most frequent among women (Brazil, 2009).

Clinic pathological parameters have been validated and serve as a guide for the use of systemic therapy and prognostication. These include tumor size, lymph node stage and histological grade, histological type and the patients' age (Lacroix et al. 2004), molecular profile and response to therapy (Rakha et al. 2009). The incidence and prevalence of most cancers increase with age (Fulop et al., 2010) and our results are in accordance to that. In the present study, the age range of 21 women breast cancer patients was 40 – 76 years old and the median age was 60 years.

Estrogen is a growth factor that stimulates cell proliferation and his effects are mediated through estrogen receptors (ER) (Ito et al, 2010). It is known that approximately 70% of breast cancers are known to express estrogen receptor (ER) alpha and are considered to be hormone-dependent (Ciocca and Elledge, 2000). In accordance with this study, in our cohort 61.9% of the patients expressed estrogen and progesterone receptors.

Regarding the histological classification, this study showed 95.24% of patients with invasive ductal carcinoma (IDC), which is in agreement with data obtained by Harris and Solin (2000), who observed incidence of 47-79% in CDI and 2-15% of invasive lobular carcinoma (CLI) in patients with breast cancer.

Our study found that 55.56% of patients had tumors between 2.1 to 5.0 cm. Tumor size is directly related to the risk of recurrence. The tumors of smaller size are invariably associated with a better prognosis for both overall survival and for disease-free survival (Abreu and Koifman, 2002) and the larger its size, the greater the chances of the existence of metastatic involvement of regional lymph nodes (Farley and Flannery, 1989, Palmer et al, 1982; Valagussa et al, 1978).

The proliferation of malignant breast epithelial cells is regulated by various stimuli including cytokines and growth factors, thus the variants of those genes may modify the individual breast cancer risk (Lee et al, 2005). The recent results have revealed that gain or loss of TGF- β signaling in carcinoma cells can promote metastasis through carcinoma cell derived TGF- β dependent host-tumor cell interactions in vivo (Bierie and Moses, 2009).

The gene encoding for TGF- β 1 is located on chromosome 19q13.1 and a T29C transition that results in a Leu10Pro substitution in the signal peptide sequence in this gene

has been associated with changes in circulating levels of TGF- β . The TGF- β 29C allele leads to significantly higher serum levels of TGF- β (Yokota et al, 2000; Ziv et al, 2001), is hypothesized to reduce breast cancer risk (Ziv et al, 2001), and has been examined in many previous breast cancer studies (Ziv et al, 2001; Kaklamani et al, 2005; Dunning et al, 2003; Jin et al, 2004; Krippel et al, 2003; Le Marchand et al, 2004; Lee et al, 2005; Shin et al, 2005; Hishida et al, 2003). It has been hypothesised that polymorphisms that affect the level of expression of this cytokine may alter an individual's susceptibility to cancers including breast (Tang et al, 1998).

Epidemiological studies found no significant association with the risk of breast cancer (Krippel et al., 2003; Le Marchand et al., 2004; Jin et al., 2004), although Hishida et al., (2003) reported that CC genotype was significantly associated with reduced risk of breast cancer in comparison with the TT genotype among Japanese women. Le Marchand et al, (2004) similarly found no significant association between TGF- β T29C polymorphism and invasive postmenopausal breast cancer in Caucasian, African-American, and Japanese in a large case-control study nested in the multiethnic cohort. In this work, it was verified prevalence of heterozygous genotype TC (Leu/Pro) (61,91%) and CC (Pro) homozygous genotype was observed only in two patients (9,52%). In reason of smaller sample number, in this work, we did not realize a case-control study association, and we only compare TGF- β polymorphisms between patients and clinic pathological and expression dates.

The genotype-phenotype relationship of T29C polymorphism has not been consistent in all ethnic groups (Dunning et al., 2003; Grainger et al., 1999; Hinke et al., 2001). Although genetic studies reveal a high degree of racial admixture in all ethnic groups in Brazil, the majority population of this study was composed of Caucasian individuals, and probably it may be explaining by the different immigration patterns that occurred in the various regions of the country, included the South region, and were we have a predominant Caucasian population.

In this study, there was no association with TGF- β polymorphism T869C with clinicopathological characteristics (Table 1) but, a trend of association of the homozygous TT genotype with hormones receptors was verified (Figure 3).

Proline homozygotes patients have been found to have increased serum levels of TGF- β (Grainger et al, 1999; Yokota et al, 2000). These results were compatible with our study, in which CC patients presented a higher expression of TGF- β mRNA expression (Figure 6).

It is known that overexpression of TGF- β by both tumor and stromal tissue can facilitate the development of metastases, mainly by TGF- β stimulated angiogenesis and

increased tumor cell motility (Dumont and Arteaga, 2000). According to literature, TGF- β has been shown to suppress the anti-tumor activity of T-cells, NK cells, neutrophils, monocytes and macrophages that are known to have a significant role in the regulation of tumor progression (Li et al., 2006; Wrzesinski et al., 2007). Carcinomas often secrete excess TGF- β and respond to it by enhanced invasion and metastasis. Therapeutic approaches should aim to inhibit the TGF- β induced invasive phenotype, but also to retain its growth-inhibitory and apoptosis-inducing effects (Akhurst and Rik Derynck, 2001).

Murray et al (1993) found that patients with high levels of TGF- β mRNA expression levels have a longer disease-free interval. The apparently opposite relationship between TGF- β and disease progression suggests that this gene may have different effects on breast cancer progression depending on the stage of the disease (Mu et al, 2008). In this work, there was a significant association between lower expression of TGF- β and increased tumor size ($p = 0.025$) $\rho(\rho= 0.484)$ (Figure 4) as well as low expression of TGF- β and increased lymphatic involvement ($p = 0.033$) $\rho(\rho= 0.484)$ (Figure 5). The risk of axillary lymph nodes being compromised is directly proportional to tumor size. Tumors up to 1 cm in diameter show the average probability of 20% to 30% of involvement by the disease, and ductal tumors with high histological grade can double the percentage of lymph node involvement (Carter et al, 1989; Fentiman et al, 1996).

It has been shown that loss of TGF- β signaling within the carcinoma cell can suppress expression of chemokines that facilitate tumor-myeloid cell interactions to promote metastasis (Bierie and Moses, 2009). The results from Zhao et al (2010) suggest a link between TGF- β and CXCR4 expression in MCF-7 human breast cancer cells, which may be one of the mechanisms of TGF- β mediated enhancement of metastatic potential in breast cancer cells. Although CXCR4 mRNA expression no significant difference was observed according to any clinic pathological features analyzed. Our work, observed for the first time, a positive correlation with TGF- β and CXCR4 expression in breast tumor tissue ($p=0.020$) (Figure 7).

The transforming growth factor beta pathway potently regulates tumor initiation, progression and metastasis. In the early years it was shown that TGF- β had the ability to suppress tumorigenesis through inhibition of cell cycle progression and induction of apoptosis. The cytostatic and apoptotic impact of TGF- β as a tumor suppressor was balanced by observations clearly demonstrating that TGF- β could promote tumor progression through suppression of immune surveillance (Bierie and Moses, 2009).

However, it has now been shown that loss of carcinoma cell responsiveness to TGF- β stimulation can also promote metastasis. Interestingly, enhanced metastasis in the absence of a carcinoma cell response to TGF- β stimulation has been shown to involve increased chemokine production resulting in recruitment of pro-metastatic myeloid derived suppressor cell populations to the tumor microenvironment at the leading invasive edge (Bierie and Moses, 2010). So, the observations suggest that it functions as a tumor suppressor or tumor promoter depending on the context of stimulation. While the impact of TGF- β on the carcinoma cell is significant, it is now generally accepted that primary and metastatic carcinoma progression is regulated by an intricate network of host-tumor cell interactions. However, many of the early studies were not able to control for local and systemic influences of exogenous TGF- β expression in the mammary tumor microenvironment (Bierie et al., 2008).

Lymphocytes, including T cells, Tregs, and natural killer (NK) cells, and their cytokine release patterns are implicated in breast cancer primary prevention and recurrence. Cancer prognosis may be related to the immune system functional status (Standish et al. 2008). During the past decade, insights have been gained regarding mechanisms underlying the dynamic interplay between immune cells and tumor progression. The accumulated data indicate that the outcome of an immune response toward a tumor is largely determined by the type of immune response elicited.

Various studies have indicated a positive correlation of VEGF expression with tumor vascularity and malignancy. Activated regulatory T cells (Tregs) release excessive levels of TGF- β has been suggested, which indirectly induces VEGF expression and leads to increased vascularity and tumor progression. It is speculated that more investigations with comprehensive information on potential confounders and comprehensive genotyping data need to be conducted to conclude about the role of TGF- β in breast cancer development. Since invasion, size and vascularity are prognostic parameters in breast cancer, the finding of a positive correlation between TGF- β and CXCR4 expression, an inverse correlation with tumor size in TGF- β expression and an association between low expression of TGF- β with increased lymph node involvement in itself, suggests a role for these genes as a progression markers for breast carcinoma.

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Regulatory T cells and breast cancer: implications for immunopathogenesis?

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Kenneth V. Honn, Ph.D.
Distinguished Professor
Dept. of Radiation Oncology &
Dept. of Pathology
Wayne State University
431 Chemistry Bldg.
Detroit, MI 48202 USA

Phone: 313-577-1018

Fax: 313-577-0798

Web Page: <http://www.cancerbiology.med.wayne.edu>

Regulatory T cells and breast cancer: implications for immunopathogenesis

Title running head: **Regulatory T cells and breast cancer**

§Corresponding author

Profa Dra Maria Angelica Ehara Watanabe (PhD),

Departamento de Ciências Patológicas, Centro de Ciências Biológicas, Universidade Estadual de Londrina, Campus Universitário - Rod. Celso Garcia Cid (PR 445) Km 380 – CEP 86051-970 – Londrina, PR, Brasil.

Telefone/ Fax: +55 (43) 3371-5728.

E-mail: maewat@uel.br

maewat@pesquisador.cnpq.br

Regulatory T cells and breast cancer: implications for immunopathogenesis

Abstract

Current understanding of the role of several cancer risk factors is more comprehensive, as reported for a number of sites, including the brain, colon, breasts and ovaries. Despite such advances, the incidence of breast cancer continues to increase worldwide. Signals from the microenvironment have a profound influence on the maintenance or progression cancers. Although T cells present the most important immunological response in tumor growth in the early stages of cancer, they become suppressive CD4⁺ and CD8⁺ regulatory T cells (Tregs) after chronic stimulation and interactions with tumor cells, thus promoting rather than inhibiting cancer development and progression. Tregs have an important marker protein that is FoxP3, though it does not necessarily confer a Treg phenotype when expressed in CD4⁺ T lymphocytes. High Treg levels have been reported in peripheral blood, lymph nodes and tumor specimens from patients with different types of cancer. 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 interleukin. In this review, we present the molecular and immunological aspects of Treg cells in metastasis of breast cancer.

Keywords: breast cancer, metastasis, Tregs, FoxP3

Introduction

Breast cancer is the most common female cancer and annually more than one million new patients are diagnosed worldwide [1]. Breast cancer incidence has increased steadily in the developed countries over the past few decades, but the mortality caused by breast cancer has decreased in recent years, partly because of improved screening techniques, surgical and radiotherapy interventions, understanding of the pathogenesis of the disease and the use of traditional chemotherapies in a more efficacious manner [2].

Breast cancer shows some distinctive features in terms of age-specific incidence rates [3] and comprises a remarkably diverse group of diseases in terms of presentation, morphology, biological characteristics, clinical behavior [4], molecular profile and response

to therapy [5]. The degree of cellular and molecular heterogeneity in breast cancer and the large number of molecular events involved in controlling cell growth, differentiation, proliferation, invasion and metastases [6] emphasize the importance of studying multiple molecular alterations in concert [7-14]. Clinicopathologic parameters have been validated and serve as a guide for the use of systemic therapy and prognostication. These include tumor size, lymph node stage and histological grade, vascular invasion, histological type and the patients' age and menopausal status [4].

During recent years it has become evident that a subpopulation of T cells named T regulatory cells (Tregs) plays a major role in sustaining tolerance to self-antigens. Forkhead box P3 (FoxP3)-expressing Tregs are key mediators of peripheral tolerance and suppress undesirable immune responses. It was verified that the Tregs bear 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/MHC complexes in the periphery, ensuring dominant control of self-reactive T cells [15].

Many studies have provided strong evidence that Tregs may express different surface molecules, reside at different locations and express molecule increase or reduction in the cells [16]. The host immune system plays an essential role in the immune surveillance and destruction of cancer cells [17, 18]. All solid tumors are embedded in a stromal microenvironment consisting of immune cells, such as macrophages and lymphocytes, as well as non-immune cells, such as endothelium cells and fibroblasts. In this context, the present review focuses on the establishment of Tregs within the immune response to breast cancer and its implications for immunopathogenesis.

Breast cancer and immune response

The immune response to tumors is complex. Cells of the immune system can inhibit tumor growth and progression by recognizing and rejecting malignant cells, a process referred to as immunoediting. Immune responses can also promote tumor cell growth, survival and angiogenesis by inducing oncogenic inflammation. Immunodeficiency can predispose to the development of spontaneous and virally induced cancer and established tumors often generate

immunosuppressive microenvironments that can block productive antitumor immunity, serving as a substantial barrier to effective immune therapy [19, 20].

Lymphocytes, including T cells, Tregs, and natural killer (NK) cells, and their cytokine release patterns are implicated in breast cancer primary prevention and recurrence. Cancer prognosis may be related to the immune system functional status [21].

Activation of humoral and cellular immunity may predispose to neoplastic or cancer development [22]. Emerging from these studies is an appreciation that persistent humoral immune responses exacerbate recruitment and activation of innate immune cells in neoplastic microenvironments where they regulate tissue remodeling, pro-angiogenic and pro-survival pathways that together potentiate cancer development. Studies on advances support the hypothesis that enhanced states of local humoral and innate immune activation, in combination with suppressed cellular immunity and failed cytotoxic T cell anti-tumor immunity, alter cancer risk and therefore represent powerful targets for anti-cancer immunotherapeutics [23].

During the past decade, insights have been gained regarding mechanisms underlying the dynamic interplay between immune cells and tumor progression. The accumulated data indicate that the outcome of an immune response toward a tumor is largely determined by the type of immune response elicited. A tumor-directed immune response involving cytolytic CD8⁺ T cells, Th1 cells and NK cells appears to protect against tumor development and progression. If, on the other hand, the immune response involves B cells and activation of humoral immunity and/or a Th2 polarized response, the probable outcome is promotion of tumor development and progression. This balance between a protective cytotoxic response and a harmful humoral or Th2 response can be regulated systemically by the general immune status of the individual, as well as locally by myeloid suppressor cells and T regulatory cells, and thus offers clinicians attractive targets for anticancer immune-based therapies [24].

Tregs induce immune tolerance by suppressing host immune responses against self- or nonself-antigens, thus playing critical roles in the prevention of autoimmune diseases, but they may inhibit antitumor immunity and promote tumor growth. Increasing evidence demonstrates that elevated proportions of CD4⁺ Treg cells are present in various types of cancers and suppress antitumor immunity. However, less is known about CD8⁺ Treg cells and their detrimental effects on immunotherapy directed toward cancer [25].

Antigen-induced suppressor T cells that were intensively studied in the 70s and early 80s remain to be reinvestigated in the light of recent findings. Thus, the current active research of T cell-mediated self tolerance and immune regulation is revealing Treg cell “unity” and “diversity”. Further investigation of Treg cells, natural or adaptive, will make their clinical use a reality for better control of a variety of physiological and pathological immune responses [26].

Regulatory T (Treg) cells

Tregs were described in 1995 by Sakaguchi [27], who reported this cell in the involvement in the of immune response regulation and cellular activation. Treg cells include populations that differ in phenotype, cytokine secretion profile and suppressive mechanism [28-30]. Several subsets of Treg cells have been identified and characterized, such as CD8⁺ Treg cells, CD4⁺ Treg cells and $\gamma\delta$ -TCR. Tregs have been reported in cancer and other diseases [25].

Studies have defined the cytokine transforming growth factor- β (TGF- β) as a critical regulator of thymic T cell development as well as a crucial player in peripheral T cell homeostasis, tolerance to self antigens, and T cell differentiation during the immune response [31]. Two main origins have been described for FoxP3⁺ cells, whose numerical and functional importance have yet to be clarified. The first is the thymus, where FoxP3⁺ cells are generated roughly in sync with positive selection of conventional CD4⁺ T cells. The second is the periphery, where a number of triggers induce the expression of FoxP3 in T cells. The conversion mechanism CD25⁻CD4⁺ in CD25⁺CD4⁺ in the periphery, *in vitro*, involves TGF- β in a murine model [32]. It was recently shown that indoleamine-2,3-dioxygenase (IDO) may also induce FoxP3 expression in CD4 T cells and IDO can convert human and murine CD4⁺CD25⁻ T cells to CD4⁺CD25⁺FoxP3⁺ cells [33]. CD4⁺CD25⁺ Tregs are important in the control of immune responses because of their ability to suppress T-cell proliferation and cytokine production [34].

CD4⁺ Treg cells can be further divided into naturally occurring CD4⁺CD25⁺FoxP3⁺ Tregs, antigen-induced CD4⁺CD25⁺FoxP3⁺ Treg cells and CD4⁺ FoxP3⁻ Tr1 cells [35]. Although the origin of CD4⁺ Treg cells remains largely unknown, they may arise from antigen-experienced CD4⁺CD25⁻ T naive and effector cells in the suppressive cytokine milieu

of tumor sites or expansion after antigen stimulation of naturally occurring CD4⁺CD25⁺ T cells. Unlike naturally occurring CD4⁺CD25⁺FoxP3⁺ Treg cells, Tr1 cells do not express FoxP3 and are induced in peripheral tissues by a major histocompatibility complex (MHC)/peptide stimulation in the presence of IL-10. They suppress immune responses through a cytokine-dependent mechanism [36, 37].

Although many authors consider FoxP3 protein the most important Tregs marker [32, 38], it does not necessarily confer a Treg phenotype when expressed in CD4⁺ T lymphocytes [39, 40]. Using specific anti-FoxP3 monoclonal antibodies, it was showed previously that only approximately half the CD4⁺CD25⁺ population expressed FoxP3, a minority of FoxP3⁺ cells lacked CD25 expression and a small number were CD8⁺ [41]. Many factors including histones, chromatin remodeling enzymes, RNA binding proteins, molecular chaperones and transcription factors may interact directly or indirectly with FoxP3 in a dynamic manner in response to extracellular stimuli [42].

FoxP3 is a member of the forkhead/winged family of transcription factors and when it acts through NFAT (nuclear factor of activated T lymphocytes) has been postulated to control key genes to specifically drive Treg development [43].

Tregs have been characterized by the constitutive expression of FoxP3, glucocorticoid-induced TNFR family-related receptor (GITR), cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) and high levels of the alpha chain of the IL-2 receptor (CD25) [44]. It is known that CD127 (alpha chain of the IL-7 receptor), which are expressed in the majority of mature T cells, play an important role in their proliferation and differentiation. However, CD127 is absent in Tregs and its expression inversely correlates with Foxp3 expression, and together with the other markers, identifies over 95% of the Foxp3⁺ cells in peripheral blood [45].

It was reported evidence that the immune systems of patients with breast cancer were dysfunctional [46]. Regulatory T cells and indoleamine 2,3 dioxygenase (IDO), an immunosuppressive enzyme, are associated with more advanced disease in some cancers and may promote immunologic tolerance to tumors. Studies have shown that FoxP3⁺ cells were associated with more advanced disease in breast cancer, a finding that is proving to be true in many other cancers. IDO has been found to promote Treg differentiation and may become a suitable target to abrogate the development of T-cell tolerance and to promote an effective immune response to breast cancer [47].

The identification of IL-17 producing FoxP3⁺ Treg cells in both mice and humans suggested that Th17 and FoxP3⁺ Treg lineages were related in ontogeny. Both lineages appeared to depend on TGF- β for their differentiation and/or maintenance, and additional cytokines may determine whether they become Th17, Treg, or dual-function effector T cells [48]. IL-17 producing FoxP3⁺ regulatory T cells were identified in humans [49]. These authors verified that human CD4⁺Foxp3⁺CCR6⁻ regulatory Tregs differentiated into IL-17 producer cells upon T-cell receptor stimulation in the presence of IL-1 β , IL-2, IL-21, IL-23 and human serum. This, together with the finding that the human thymus not contain IL-17-producing Tregs, suggested that the IL-17⁺FoxP3⁺ Tregs were generated in the periphery. IL-17-producing Tregs may play critical roles in antimicrobial defense, while controlling autoimmunity and inflammation.

It has been strongly suggested that the increase in functional Tregs in cancer patients was a response to the process of malignant transformation [50]. It is of interest to know whether Treg cell expansion in solid tumors is also accompanied by the expansion of naive Tregs and whether there are differences in different compartments such as the blood, secondary lymphoid organs, and bone marrow or at the tumor site.

Tumor-derived CD4⁺ Treg cells have been extensively studied in many different types of cancer. This notion is further supported by the fact that antigen-specific CD4⁺ Tregs at tumor sites may significantly suppress immune responses, leading to immune tolerance of tumor cells. Despite the importance of immune cells such as T cells in the immunosurveillance and control of tumor growth in the early stages of cancer, they become suppressive CD4⁺ and CD8⁺ regulatory Tregs after chronic stimulation and interactions with tumor cells, thus promoting rather than inhibiting cancer development and progression [51]. Neither tumor Treg nor naive Treg can suppress antitumor immunity at the effector phase of the immune response induced by adoptively transferred tumor-primed CD4⁺ T cells. Therefore, tumor Tregs potentially abrogate tumor-specific CD8⁺ T cell responses in tumor-draining lymph nodes, thereby suppressing antitumor immunity at the early stage of the immune response induced by adoptively transferred tumor-primed CD4⁺ T cells [52].

Regulatory T cells: implications in breast cancer

Interleukins and cytokines are important regulators of the aetio-pathogenesis of the majority of cancers [53]. It was reviewed the stability of a Tregs population, which can down regulate FoxP3, lose regulatory activity and, under some conditions, become memory T cells capable of recognizing self-antigens and expressing effector cell activities including the production of IL-17 and IFN gamma [54]. They concluded that the presence of these 'exTregs' in multiple inflammatory settings suggested a potential role for these cells in a variety of disease settings ranging from autoimmunity to cancer and infectious disease.

Tregs enriched in FoxP3⁺, GITR⁺, and CTLA4⁺ exert a potential to suppress effector T cells in the periphery. These cells exist in markedly higher proportions within tumor-infiltrating lymphocytes, peripheral blood lymphocytes, and/or regional lymph node lymphocytes of patients with cancer. Their frequencies are suggested to be strongly related to tumor progression and inversely correlated with the efficacy of the treatment. Treg cell depletion or blockade can enhance immune protection from tumor-associated antigens that are expressed as self antigens [55].

To determine whether intratumoral Treg accumulation and activation help in the progression of human breast carcinoma, it was analyzed the intratumoral expression of Foxp3 in invasive breast carcinoma and compared it with its level in ductal carcinoma in situ and adjacent normal tissue with the main aim of using this factor as a tumor progression marker [56]. These authors verified that a linear association of intratumoral FoxP3 expression with invasion, size and vascularity suggested a use for FoxP3, an indicator of Treg activity, as a marker of tumor progression and metastasis in breast carcinoma.

It has been investigated whether expression of FoxP3 transcripts and mature protein occurred constitutively in various tumor types and demonstrated that cancer cells of various types expressed a transcript for FoxP3 as well as the mature protein [57].

It was compared the frequency of CD4⁺CD25^{high} in the peripheral blood of cancer patients and healthy donors and demonstrated evidence of an increased CD4⁺CD25^{high} pool in the peripheral blood of cancer patients, which may be related to immunosuppression and tumor progress in cancer patients [58].

High-risk breast cancers, especially breast cancers at risk for recurrence, recruit high numbers of Tregs, suggesting a correlation with disease prognosis [59]. Analysis of human

breast cancer samples provided strong support for an important role for the FoxP3 gene in the development of breast cancer [60].

Low level FoxP3 mRNA expression was detectable in breast epithelium and breast cancer cell lines, where FoxP3 functions as a breast cancer suppressor gene that may help to understand the origin of FoxP3-expressing cells; those are breast epithelium, breast cancer cells, or Tregs. However, they demonstrated that deletion, functionally significant somatic mutations and downregulation of the FoxP3 gene were commonly found in human breast cancer samples [61]. It has been reported that FoxP3 expression was higher in tumor tissue than in normal breast tissue [62]. However, it was strongly suggested that the FoxP3 expression in breast cancer tissue indicated the tumor-infiltrating Treg cell origin. It has been suggested that the function of Foxp3 in cancer cells may depend on the nature of the breast tumor, especially concerning oncogenic pathways involved in tumor growth [63].

The clinical significance of tumor-infiltrating FoxP3-positive Tregs has been assessed in breast cancer patients with long-term follow-up [59]. These authors present the finding that high FoxP3-positive Tregs numbers represent an important marker for the identification of breast cancer patients at risk of late relapses. They concluded that the number of tumor-associated Tregs is a significant parameter for disease prognosis in both invasive and noninvasive breast tumors that can be assessed in routinely fixed tissues by immunohistochemistry to detect FoxP3 positive T cells. The authors strongly suggested that such therapy would be beneficial for a significant proportion of breast cancer patients.

A T lymphocyte inhibitory molecule named B7-H1 (also called PD-L1), expressed by antigen presenting cells, has been shown to induce T lymphocyte anergy after linking to its T lymphocyte receptor PD-1 [64]. B7-H1 has been shown to be directly involved in the protection of cancer cells from activated T lymphocytes [65]. It was investigated T cells infiltrating lymphocytes expressing the B7-H1, PD-1 and FoxP3 molecules in the microenvironment of human breast tumors and their possible association with the progression of the disease. A concurrent and abundant infiltration of different immune suppressive subsets of T lymphocytes has been shown in the microenvironment of high-risk breast cancer patients. This interesting observation suggests the development of new therapeutic modalities to target B7-H1/PD-1 and Tregs in addition to still-developing immunotherapy [66].

Studies have shown that Tregs might also be generated from T cell-derived tumor cells [67, 68]. It was suggested that Tregs are involved in tumor onset and progression in human

primary breast cancer, possibly contributing to poor prognosis of patients with breast cancer. FoxP3, IL-10, TGF β 1 and CCL22 mRNA expressions were significantly higher in cancer tissue than in normal tissue. FoxP3 and IL-10 mRNA expressions were significantly upregulated in progesterone receptor -negative or HER2-positive tumors [62].

In breast cancer, the Treg number is increased in the peripheral blood of breast cancer patients [69- 71] and they are present within the primary tumors [69]. A recent study demonstrated a significant intratumoral infiltration of FoxP3⁺ Tregs in high-risk breast cancer patients and those at risk of late relapse [59].

The level of mRNA expression of CTLA-4 in normal and breast carcinoma tissues has been demonstrated and showed statistically increased levels of the gene transcription in patients that correlated with disease progression [72]. Some researchers has evaluated CTLA-4 and FoxP3 transcripts, as acceptable indicators of Tregs, in the peripheral blood from women with breast cancer and found that these transcripts significantly increased even in the early stages of breast cancer [73].

By taking advantage of a highly conserved FoxP3 sequence, it was genotyped three haplotype-tagging single-nucleotide polymorphisms that covered 40 kb around the FoxP3 gene region and verified that FoxP3 was a biologically relevant gene in breast cancer pathogenesis, but germline variation in their study was not meaningfully associated with risk of the disease [74].

Possible functions of the T cells in disseminated breast cancer

Adding to the complexity of metastasis, this process often follows characteristic organ distribution patterns that reflect inherent differences within the disseminating cells of distinct tumors [75, 76].

Some authors have reported that the presence of immune cells in breast tumors is unable to counteract cancer and may even contribute to tumor progression. Metastatic cancer is associated with an expansion of peripheral blood CD4⁺CD25^{high}FoxP3⁺GITR⁺CD152⁺ Tregs whose immunosuppressive properties do not differ from those of healthy subjects [77].

It has been suggested that homeostatic mechanisms governing the peripheral blood count of FoxP3⁺ CD4⁺ T cells differ fundamentally from those governing the total CD4⁺ T

cell count; this different regulation would explain, for example, why the average absolute CD4 count in patients with metastatic disease or those post-chemotherapy was lower than that for healthy volunteers, yet the absolute count of FoxP3⁺ CD4⁺ T cells was not. Regulatory and non- Tregs might be regulated differently by cytokines such as IL-2 or exhibit differential susceptibility to the effects of chemotherapy or macroscopic tumor burden [78].

In 2007, it was determined the number and functional status of CD4⁺CD25^{high} Tregs in blood samples from patients with metastatic carcinoma and they found Treg numbers were significantly higher in patients with metastatic cancer compared to healthy donor [77].

In 2009, some authors studied patients diagnosed with invasive breast carcinoma who underwent primary systemic chemotherapy followed by definitive surgery and examined the correlations between the number of tumor-infiltrating FoxP3-positive cells during primary systemic chemotherapy and therapeutic effects in patients with breast cancer. They demonstrated that lymph vessel invasion was prominent in the group with a high number of FoxP3 infiltrates [79].

It has been suggested the use of FoxP3 as a novel, independent molecular marker of breast carcinoma outcome, with a significant impact on important outcome measures for breast carcinomas. FoxP3 expression in tumors was associated with worse overall survival probability and the risk increased with increasing FoxP3 immunostaining intensity. FoxP3 was also a strong prognostic factor for distant metastases-free survival but not for local recurrence risk [80].

Additional insight was provided into the regulatory mechanisms responsible for immunosuppression in human cancer, which may facilitate local tumor growth and metastasis. Hematogenic metastasis often represents the fatal step during the course of malignancy, which may be significantly enhanced by the suppression of blood-borne immunosurveillance mechanisms. Treg depletion may become a successful anticancer strategy and Treg manipulation in terms of their frequency and functional activity should be added to the therapeutic to enhance tumor immunity in humans [71].

Chemokines and their receptors are involved in the control of lymphocyte, a critical component of systemic immunity. G-protein-coupled receptor (CXCR4) is a receptor of considerable biological significance and its numerous functions suggest that it is involved in diverse development processes. It was demonstrated that samples of peripheral blood cells of

stage II samples from breast cancer patients revealed higher CXCR4 expression than the controls and other stages [81]. CXCL12 is a chemokine that binds to a CXCR4. CXCL12 is expressed in various tumors and is considered to play an important role in tumor growth and invasion [82]. Authors has investigated CXCL12 expression in human malignant mesothelioma, the chemotactic effect of CXCL12 derived from mesothelioma and CXCR4 expression in mesothelioma tissues in relation to regulatory T cells. CXCL12 was expressed in mesothelioma cell cytoplasm from all patients, but it was not expressed in the control group. These findings suggested that CXCL12 contributed to tumor-related inflammation by inducing the accumulation of CXCR4-expressing cells with regulatory T cell markers around mesothelioma [83].

It was observed that Tregs expressed chemokine receptor CCR4 and showed demonstrable chemotactic responses to the CCR4 ligands CCL22 and CCL17 [84]. On accumulation of regulatory T cells in cancer, Tregs may be attracted by various chemokines (CCL5, CCL17, CCL22, CXCL12) to the tumor site. Cancerous cells and/or by standing tumor-associated macrophages and myeloid-derived suppressor cells secrete these chemokines of which Tregs possess the corresponding receptors as CCR4, CCR5 and CXCR4 [85]. The increased of frequency of a new Treg subset was reported, CCR6⁺ Tregs, correlated positively with the poor survival of breast cancer patients. It suggested that the CCR6⁺ subset of Tregs might be mainly responsible for long-term immunosuppression in the tumor environment. However, successive broad screening approaches to the role of CCR6⁺ Tregs in other tumor hosts will be worthwhile to further substantiate these initial results, which might throw a novel insight on the role of the resident unique subset of Tregs in the tumor mass and provide helpful thoughts for the designing of Treg-based immunotherapy strategy against tumors in the future [86].

Various studies have indicated a positive correlation of VEGF expression with tumor vascularity and malignancy. Activated Tregs release excessive levels of TGF- β 1 has been suggested, which indirectly induces VEGF expression and leads to increased vascularity and tumor progression. This implies that FoxP3 levels, an indicator of Treg activity, might also be an indicator of breast tumorigenesis. Since invasion, size and vascularity are prognostic parameters in breast cancer, the finding of a positive correlation between FoxP3 expression and these parameters suggests a role of FoxP3 as a progression marker for breast carcinoma to an aggressive tumor phenotype [56].

It has been investigated FoxP3 protein expression in breast cancer by immunohistochemistry and demonstrated that high numbers of FoxP3-positive Tregs were present in high-grade tumors at increased risk of relapse [59].

Breast cancer cells disseminate through the body by direct extension, lymphatic channel invasion and circulation through blood vessels [87]. Breast cancer preferentially spreads to the bones, lungs, liver, and brain, whereas prostate cancer almost exclusively colonizes the bones [88]. Lymph node involvement remains the most influential prognostic factor in breast cancer progression. The presence of metastatic tumor cells in a lymph node is associated with specific alterations in the T cell population [89]. Sentinel lymph nodes are the nodes nearest to a primary tumor on the direct lymphatic drainage pathway of the breast and are the typical site of earliest metastasis. It was indicated that FoxP3⁺ Tregs increased in the microenvironment of sentinel lymph nodes along with pathologically undetectable micrometastasis and were an independent prognostic predictor in patients with node-negative breast cancer [90]. In this context, it was observed that Treg response was induced at the micrometastasis level and persisted during metastasis progression in sentinel nodes in breast cancer patients [91].

TGF- β , which is a kind of cytokine produced by Tregs, has been implicated in tumor progression. The TGF- β pathway has been implicated in many of these metastatic processes and has been shown to dramatically impact the ability of tumor cells to spread throughout the body [92-95].

Tregs were selectively recruited within lymphoid infiltrates and activated by mature dendritic cells likely through recognition of tumor-associated antigens presentation, resulting in the prevention of effector T-cell activation, immune escape and ultimately tumor progression [96]. It has been suggest that FoxP3 was expressed in breast cancer cells and the expression level was associated with patient survival. They found that FoxP3 expression was associated with overall and distant metastasis free survival but not with local relapse and therefore, the authors suggested that FoxP3 expression might be related to the metastatic potential of the tumor rather than to suppression of a specific immune response [80].

It has been hypothesized that Tregs accumulation in tumor tissue would increase in parallel with tumor progression. They found higher expression of FoxP3 mRNA in tumor tissue than in normal breast tissue and it was observed even at the ductal carcinoma *in situ*

stage and persisted at the T1 and T2,3 stages, indicating that Tregs accumulation in tumor tissue was an early event in tumor development and progression [62].

Interestingly, the FoxP3 transcription factor up- or downregulates a large number of genes and has been recently reported to be expressed in tumor cells. Furthermore, FoxP3 binds to the gene region upstream of the transcriptional start site of CCR7 and CXCR4 [97], two chemokine receptors recently reported to play an important role in cancer invasion and metastasis [98, 99]. Thus, FoxP3 expressed in breast cancer cells might influence metastasis development by modulating the expression of these chemokine receptors or of other genes encoding cell surface or secreted molecules that alter tumor cell response to the environment [80].

Nevertheless, the finding that FoxP3 can be expressed by not only tumor-infiltrating Tregs but also by tumor cells has two important implications. First, caution needs to be taken when interpreting gene expression data on FoxP3 expression in tumors. Increased levels of FoxP3 mRNA expression may be a result of not only an increased influx of Tregs but also the increased expression of FoxP3 directly in tumor cells. This understanding has significant importance for developing assays on the basis of FoxP3 for prognosis or drug monitoring. Second, we need to recognize that FoxP3-targeted therapy may need to be targeted at not only Tregs but also FoxP3-positive tumor cells, although the role of FoxP3 in regulating tumor cell growth remains to be clarified. The expression of FoxP3 in tumor cells indicates that FoxP3-targeted drugs must be able to penetrate the tumor bed, which is much more challenging than depleting FoxP3 in the periphery [100]. A schematic model of Tregs in breast cancer dissemination is represented depicting their possible interplay with breast cancer cells in the microenvironment and the factors recruiting them to the cancer (Figure 1).

The discovery of the FoxP3 transcription factor as a central molecular determinant of Treg differentiation and function has made the complex biology of these cells, including maintenance of immunological tolerance to "self" and regulation of immune responses to pathogens, commensals and tumors has become a focus of intense investigation. The FoxP3 gene plays a crucial role in Treg the generation whereas the over expression of FoxP3 results in severe immunodeficiency. Tregs may play an important role in breast cancer immunopathology due to their potent suppressive activity of both T cell activation and effector function. Comprehensive analysis of immune effector functions at different stages of tumor metastasis is fundamental to the design of effective immune intervention. Although it is

known that the clinical behavior of tumors depends on the relationship between tumor cells and the host, there are reports involving molecular research which identified tumor-derived markers, but little is known about the predictive potential of host factors and their potential role in breast cancer pathogenesis. The precise mechanisms to understand how Tregs suppress immune cell functions remain unclear, whether inhibition is through cell-cell contact or by indirect inhibition through the involvement of anti-inflammatory mediators in the microenvironment. Tregs cells can avoid the anti-tumor activity of immune effector cells in breast cancer tissue, resulting in poor prognosis of breast cancer patients. Tregs exhibit potent immunosuppressive functions and are known to infiltrate primary tumors and draining lymph nodes. TGF- β 1, which is one kind of cytokine produced by Treg cells, has been implicated in tumor progression. Although TGF- β 1 has been reported as a multifunctional growth factor, in breast cancer, this factor could induce the expression of the vascular endothelial growth factor, which is one of the most selective and potent angiogenic factors known, therefore, the TGF- β pathway has been implicated in many of these metastatic processes. It has been also verified expression of FoxP3 in the tumor cells with expression of chemokine receptor as CXCR4 and CCR7 which has been reported to play an important role in cancer invasion. It has inserted significance of Tregs implicated in carcinogenesis and efforts towards the development of anticancer approaches for inhibiting of the expression of FoxP3 by tumor-associated Tregs. Comprehensive analysis of immune effector functions at different stages of tumor metastasis is fundamental to the design of effective immune intervention. It could be suggested that Tregs numbers could constitute an important prognostic factor for patients with breast cancer treated with primary systemic chemotherapy, and FoxP3-positive cells in tumors could be a novel therapeutic target that could improve outcomes for such patients.

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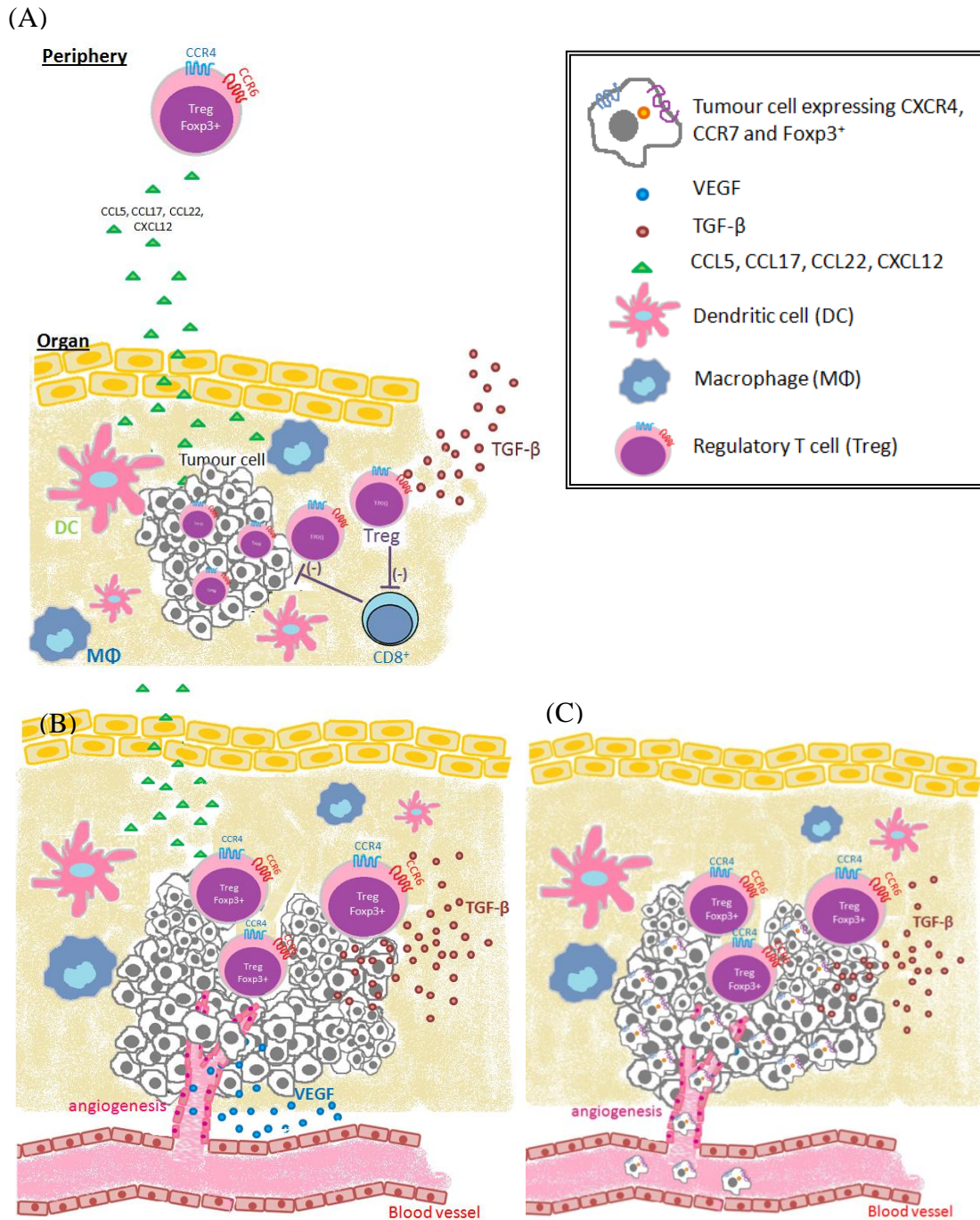


Figure 1. Possible role of Tregs in the tumor microenvironment. (A) Macrophages, dendritic cells and tumor cells in the environment of tumour development, secrete chemokines such as CCL5, CCL17, CCL22 and CXCL12 which attract peripheral regulatory T cells (Treg) to the tumour environment. (B) Tregs secrete TGF-β which plays important functions in endothelial cells and tumour cells. TGF-β induce angiogenesis by secreting VEGF from remaining endothelial cells. (C) Tumour cells express Foxp3+ and chemokines receptors, such as CXCR4 and CCR7. These modified cells (tumour cells) became able to reach the blood vessel.

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