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MARINA OKUYAMA KISHIMA

**POLIMORFISMO GENÉTICO, EXPRESSÃO GÊNICA E  
PROTEICA DO RECEPTOR CXCR4:  
IMPLICAÇÕES NA PATOGÊNESE DO CÂNCER DE MAMA E  
POSSÍVEL CORRELAÇÃO COM O GENE *TP53***

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Orientadora: Profa. Dra. Maria Angelica Ehara Watanabe

Coorientadora: Profa. Dra. Roberta Losi Guembarovski

Londrina  
2017

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

Kishima, Marina Okuyama.

Polimorfismo genético, expressão gênica e proteica do receptor CXCR4: implicações na patogênese do câncer de mama e possível correlação com o gene TP53 / Marina Okuyama Kishima. - Londrina, 2017.

73 f.

Orientador: Maria Angelica Ehara Watanabe.

Coorientador: Roberta Losi-Guembarovski.

Tese (Doutorado em Patologia Experimental) - Universidade Estadual de Londrina, Centro de Ciências Biológicas, Programa de Pós-Graduação em Patologia Experimental, 2017.

Inclui bibliografia.

1. câncer de mama, polimorfismo genético, CXCR4, TP53 - Tese. I. Watanabe, Maria Angelica Ehara. II. Losi-Guembarovski, Roberta. III. Universidade Estadual de Londrina. Centro de Ciências Biológicas. Programa de Pós-Graduação em Patologia Experimental. IV. Título.

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## **AGRADECIMENTOS**

Às minhas orientadora e co-orientadora, Profa. Dra. Maria Angelica Ehara Watanabe e Profa. Dra. Roberta Losi Guembarovski, respectivamente, pela preciosa orientação, dedicação e profissionalismo. Agradeço também pela amizade nesta etapa tão especial. Meu muito obrigada!!!

À todos os membros do “Laboratório de Estudos e Aplicações de Polimorfismos de DNA”, coordenado pela Profa. Dra. Maria Angelica Ehara Watanabe, em especial à Dra. Marla Karine Amarante, por estarem sempre dispostos a ajudar e pela maneira como trabalham em equipe.

À banca avaliadora deste trabalho, Profa. Dra. Ana Cristina da Silva do Amaral Herrera, Profa. Dra. Edna Vissoci Reiche, Profa. Dra. Karen Brajão de Oliveira e Profa. Dra. Carolina Batista Ariza, por terem aceitado participar deste momento tão importante.

À banca avaliadora da qualificação, Profa. Dra. Karen Brajão de Oliveira e Profa. Dra. Carolina Batista Ariza, pelas valiosas sugestões e contribuições na primeira etapa.

Ao Dr. Walter Jorge Sobrinho da Ginecomed clínica de Mastologia e ao setor de Patologia do Hospital do Câncer de Londrina, pela grande colaboração na obtenção das amostras e dos dados clínicos das pacientes.

Aos médicos patologistas Dra. Dora Grimaldi, Dr. Kazuhiro Ito e Dra. Alda Losi Guembarovski, pela grande ajuda e incentivo em vários momentos desta caminhada.

À técnica do Laboratório de Patologia do Hospital Universitário de Londrina, Lucia, que contribuiu muito na confecção das lâminas e na etapa de imunohistoquímica. Sem a sua ajuda este trabalho não teria sido realizado.

A todas as pacientes e controles que contribuíram com amostras e dados clínicos, o meu muito obrigada, com todo carinho e respeito.

Ao programa de Pós-graduação em Patologia Experimental da Universidade Estadual de Londrina, por todo o suporte durante a realização deste trabalho.

A Universidade Estadual de Londrina, CAPES e CNPq pelo suporte financeiro.

Gostaria de agradecer de forma muito especial a toda a minha família. Aos meus pais, com saudades, por tudo que representam na minha vida. Em especial gostaria de expressar meus sinceros agradecimentos ao meu marido Paulo Kishima, e aos meus filhos Mariana e Luís Felipe, por todo o apoio, compreensão, carinho e pela sabedoria e harmonia com que sempre superamos os momentos difíceis. Meu eterno amor e gratidão.

A todos aqueles de alguma forma contribuíram para a realização deste trabalho gostaria de deixar o meu muito obrigada!!!

A Deus meu Mestre e Mentor, pela oportunidade desta evolução pessoal e espiritual.

KISHIMA, Marina Okuyama. **Polimorfismo genético, expressão gênica e proteica do receptor CXCR4: implicações na patogênese do câncer de mama e possível correlação com o gene TP53.** 2017. 73 f. Tese de Doutorado (Programa de Pós-graduação em Patologia Experimental) – Universidade Estadual de Londrina, Londrina, 2017.

## RESUMO

Os tumores de mama são caracterizados por um padrão metastático distinto, envolvendo linfonodos, medula óssea, fígado e pulmão. A migração das células tumorais metastáticas apresenta muita semelhança com o tráfego de leucócitos. Tem sido relatado que estas podem ser guiadas através da circulação até órgãos distantes que expressam quimiocinas, pois tais células possuem receptores específicos, como o CXCR4 (*C-X-C motif chemokine receptor 4*). Este receptor vem sendo implicado na disseminação de tumores malignos, sendo visto como um marcador promissor em vários tipos de neoplasias, incluindo as da mama. O objetivo do presente estudo foi investigar a influência do polimorfismo genético de CXCR4 (C/T rs2228014) sobre sua própria expressão gênica e proteica, bem como em relação a parâmetros prognósticos da doença, em 74 amostras de pacientes com câncer de mama geral. Tivemos ainda como objetivo avaliar um polimorfismo (rs1042522) no gene *TP53*, procurando estabelecer uma correlação com a expressão gênica de CXCR4, bem como com parâmetros prognósticos em uma amostra de 33 pacientes com câncer de mama, luminal/HER2 (-). DNA e RNA foram extraídos a partir de tecido tumoral e normal obtidos por ressecção cirúrgica utilizando-se kits específicos. A genotipagem de CXCR4 foi realizada por reação em cadeia da polimerase seguida de análise de fragmentos de restrição (RFLP-PCR). A expressão gênica foi avaliada por PCR quantitativa em Tempo Real (q-PCR) e a expressão proteica por imunohistoquímica. As frequências genotípicas das pacientes demonstraram que 88,24% apresentaram genótipo homocigoto CC e 11,76% apresentaram genótipo heterocigoto CT. Nenhuma diferença significativa na distribuição dos genótipos foi observada de acordo com as características clinicopatológicas (grau histológico, grau nuclear, comprometimento de linfonodo, receptor de estrogênio e/ou progesterona, p53, Ki67 e superexpressão do oncogene HER2). Em geral, as amostras de câncer de mama apresentaram maior expressão de CXCR4 (5,7 vezes) em relação ao RNAm de mama normal, mas não foram observadas diferenças significativas em relação à variante alélica polimórfica, quanto à expressão proteica e também em relação aos parâmetros acima citados. No ensaio imunohistoquímico, foi observada uma marcação citoplasmática mais intensa de CXCR4 nas amostras tumorais em relação ao tecido normal de mama ( $p < 0,01$ ), mas esta não foi associada ao comprometimento linfonodal, expressão gênica ou ao polimorfismo rs2228014. O estudo caso controle indicou uma associação positiva para o genótipo heterocigoto e para o portador do alelo C (modelo dominante) do gene *TP53* em relação à suscetibilidade em amostras luminal/HER2(-). Foi verificada uma correlação significativa para a variante genética (alelo C) de *TP53* em relação ao tamanho tumoral e ao índice de proliferação celular, e a expressão de CXCR4 apresentou correlação significativa com presença de metástase, nesta mesma amostra. Adicionalmente, nenhum dos genótipos de *TP53* influenciou a expressão gênica de CXCR4. De um modo geral, o presente estudo mostrou um aumento da expressão de CXCR4 nos tecidos tumorais de mama, tanto em nível gênico quanto proteico, o qual não foi influenciado pelo polimorfismo avaliado no mesmo gene. Tais resultados sugerem que este receptor pode ser um marcador promissor no contexto geral da carcinogênese mamária, em especial no que se refere ao processo de metástase, mas deve ser investigado se esse padrão se repete nos outros subtipos tumorais da mama.

**Palavras-chave:** Câncer de mama. Receptor de quimiocina. CXCR4. Polimorfismo genético. *TP53*. Expressão gênica. Imunohistoquímica.

KISHIMA, Marina Okuyama. **Genetic polymorphism, genic and protein expression of CXCR4 receptor: implications in breast cancer pathogenesis and possible correlation with TP53 gene.** 2017. 73 pp. Doctoral Thesis (Postgraduate Program in Experimental Pathology) – Universidade Estadual de Londrina, Londrina, 2017.

## ABSTRACT

Breast cancer (BC) is characterized by a distinct metastatic pattern involving lymph nodes, bone marrow, liver and lung. It is known that metastatic tumor cells migration resembles the leukocyte trafficking. It has been reported that these cells can be guided through the circulation to distant organs that express chemokines, since such cells have specific receptors, such as CXCR4 (C-X-C motif chemokine receptor 4). This receptor has been implicated in the spread of malignant tumors, being a promising marker in several types of neoplasia, including BC. The objective of the present study was to investigate the influence of CXCR4 (C/T rs2228014) polymorphism on its own gene and protein expressions, as well as in relation to disease prognostic parameters, in 74 BC general samples. We also aimed to evaluate another polymorphism (rs1042522) in the *TP53* gene, searching to establish a correlation with CXCR4 expression, as well as with disease prognostic parameters in 33 luminal/HER2- BC samples. DNA and RNA were extracted from tumor and normal tissues obtained by surgical resection using specific kits. CXCR4 genotyping was performed by polymerase chain reaction followed by restriction fragment analysis (PCR-RFLP). Gene expression was assessed by Real-time quantitative PCR (q-PCR) and protein expression by immunohistochemistry. Genotype frequencies of the patients showed that 88.24% presented CC homozygous and 11.76% presented CT heterozygous genotypes. No significant difference in genotype distribution was observed according to clinicopathological features (histological grade, nuclear grade, lymph node involvement, estrogen and/or progesterone receptor, p53 staining, Ki67 staining and HER2 overexpression). In general, BC samples showed higher expression of CXCR4 (5.7 times) than normal adjacent tissue, but no significant differences were observed in relation to allelic variant, protein expression or prognostic parameters above mentioned. In immunohistochemical assay, a more intense CXCR4 cytoplasmic staining was observed in tumor tissue in relation to normal adjacent tissue ( $p < 0.01$ ), but it was not associated with lymph node involvement, gene expression or CXCR4 polymorphism. Case control study indicated a positive association for *TP53* heterozygous genotype and for C allele carriers in relation to luminal/HER2(-) BC susceptibility. A significant correlation was found for a genetic variant (C allele) of *TP53* in relation to tumor size and cell proliferation index parameters. CXCR4 expression also showed a significant correlation with metastasis parameter, in this same luminal/HER2- BC sample. In addition, none of the *TP53* genotypes influenced *CXCR4* gene expression. Overall, the present study showed an increase of CXCR4 expression in BC tissues, both at the gene and protein levels, which was not influenced by the evaluated polymorphism at the same gene. All in all, these results suggest that this receptor may be a promising marker in the general context of breast carcinogenesis, especially regarding the metastasis process, but it should be investigated whether this pattern repeats in the other tumor subtypes of the breast cancer.

**Keywords:** Breast cancer. Chemokine receptor. CXCR4. Genetic polymorphism. *TP53*. Gene expression. Immunohistochemistry.

## LISTA DE ABREVIATURAS E SIGLAS

3'UTR	Região 3' não traduzida
Arg	Arginina
Bcl	Endonuclease de restrição Bcl
BRCA1	Câncer de mama 1
BRCA2	Câncer de mama 2
CEPs	Células endoteliais progenitoras
C	Citosina
CDI-SOE	Carcinoma Ductal Invasivo, Sem Outras Especificações
cDNA	Ácido desoxirribonucleico complementar
CK5	Citoqueratina 5
CK6	Citoqueratina 6
C-Kit	Proto-oncogene c-Kit
CNS	Conselho Nacional de Saúde
CXCL12	C-X-C quimiocina 12
CXCR4	C-X-C receptor de quimiocina 4
DNA	Ácido desoxirribonucleico
EDTA	Ácido etilenodiamino tetra-acético
EGFR	Receptor do fator de crescimento epidérmico tipo 1
EMT	Transição Epitelial Mesenquimal
FACs	Fibroblastos associados às células tumorais
FISH	Hibridização <i>in situ</i> por fluorescência
G	Guanina
GAPDH	Gliceraldeído-3-fosfato desidrogenase
HER2	Receptor do Fator de Crescimento Epidérmico Humano tipo 2
IARC	<i>International Agency for Research on Cancer</i> (Agencia Internacional para Pesquisa sobre Câncer)
IHQ	Imunohistoquímica
IC95%	Intervalo de Confiança a 95%
INCA	Instituto Nacional do Câncer
KCL	Cloreto de potássio
Ki67	Antígeno nuclear Ki67

LEAP	Laboratório de Estudos e Aplicações de Polimorfismos de DNA
MgCl <sub>2</sub>	Cloreto de Magnésio
M-MLV	<i>Moloney Murine Leukemia Virus Reverse Transcriptase</i>
RT	(Transcriptase reversa codificado pelo vírus Moloney Murine Leukemia)
	Fator nuclear Kappa B
NF-κB	Organização Mundial da Saúde
OMS	<i>Odds ratio</i> (Razão de possibilidades)
OR	Pares de bases
pb	Poli ADP ribose polimerase
PARP	Reação em cadeia da polimerase
PCR	Reação em cadeia de polimerase, quantitativo
qPCR	Prolina
Pro	Ácido ribonucleico
RNA	Receptor de estrógeno
RE	Polimorfismo do comprimento do fragmento de restrição
RFLP	Receptor hormonal
RH	Receptor de progesterona
RP	Rotações por minuto
RPM	Polimorfismos de nucleotídeo único
SNPs	Sinal de transdução e ativador de transcrição 3
STAT3	Timina
T	Tampão Tris-Acetato-EDTA
TAE	Tumor-Nódulo-Metástase
TNM	Gene Supressor Tumoral p53 (humano)
TP53	Triplo-negativo
TN	Unidade
U	<i>Union for International Cancer Control</i> (União Internacional de
UICC	Controle ao Câncer)
	Unidade Lobular do Ducto Terminal
ULDT	<i>World Health Organization</i> (Organização Mundial da Saúde)
WHO	Xileno Cianol
XC	

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# 1. INTRODUÇÃO

## 1.1. Câncer de mama

### 1.1.1 Epidemiologia, classificação e fatores de risco

A importância mundial do câncer é inquestionável, uma vez que é a segunda maior causa de mortes no mundo, superada apenas pelas 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. De acordo com estimativas mundiais do projeto GLOBOCAN 2012, da *International Agency for Research on Cancer- IARC* e da Organização Mundial da Saúde (OMS), ocorreram 14,1 milhões de casos novos de câncer e 8,8 milhões de mortes por câncer em todo mundo em 2015 (WHO 2015).

O câncer de mama é o segundo tipo de câncer mais incidente no mundo e o que tem a maior incidência e a maior mortalidade na população feminina. Segundo os dados do Instituto Nacional do Câncer (INCA) para o Brasil, em 2016, foram estimados 57.960 novos casos, com um risco estimado de 56,09 casos a cada 100 mil mulheres. Sem considerar os tumores de pele não melanoma, esse tipo de câncer é o mais frequente nas regiões Sul (74,30/100 mil), Sudeste (68,08/100 mil), Centro-Oeste (55,87/100 mil) e Nordeste (38,74/100 mil). Na região Norte, é o segundo tumor mais incidente (22,26/100 mil), sendo o primeiro o carcinoma do colo uterino, tendo como principais motivos a falta de acesso ao exame preventivo e ao tratamento. Suas taxas de incidência variam entre as diferentes regiões do mundo, com as maiores taxas na Europa Ocidental e as menores taxas na Ásia Oriental (INCA 2016).

Na última classificação da OMS (2012), foram reconhecidos mais de 20 subtipos de câncer de mama. Os tumores epiteliais foram agrupados, com o reconhecimento de nove tipos especiais e suas variantes e 11 tipos raros de tumores mamários, além do carcinoma invasivo de tipo não especial (TNE) /carcinoma ductal invasivo sem outra especificação (CDI-SOE). Os tumores mamários invasivos, em sua maioria, cerca de 75% dos casos, são carcinomas ductais, que não apresentam padrão citológico ou arquitetural especial, sendo portanto denominados, na classificação atual, como TNE. O objetivo é não utilizar o termo “ductal”, evitando o conceito incorreto de que estes tumores são derivados exclusivamente do epitélio ductal mamário. Sabe-se que a maioria dos carcinomas ductais ou lobulares se origina da unidade lobular do ducto terminal (ULDT). Os carcinomas lobulares representam cerca de 5 a

15% dos tumores invasivos da mama e os outros subtipos raros representam menos de 10% dos casos diagnosticados por ano (Stephens, Tarpey et al. 2012). As pacientes com carcinoma invasivo de tipo não especial apresentam maior envolvimento linfático e um pior prognóstico que o verificado nas pacientes com tipos menos frequentes de carcinoma invasivo da mama (Ketterhagen, Quackenbush et al. 1984).

O estadiamento do tumor é realizado após a confirmação do diagnóstico e associado ao grau de diferenciação histológica. Estes influenciam o prognóstico 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), que se baseia na extensão anatômica da doença, considerando as características do tumor primário (T), a ausência ou presença e a extensão de metástase em linfonodos regionais (N) e a ausência ou presença de metástase à distância (M). O sistema TNM classifica as pacientes portadoras de carcinoma invasor em quatro estádios denominados I, II, III e IV. Pacientes nos estádios I e II são consideradas portadoras de doença em fase inicial, com tumores pequenos localizados na mama. No entanto, há diferenças de sobrevida entre o estágio I, em comparação com aquelas portadoras de metástases nos linfonodos axilares (estágio II). Dentro da doença no estágio II, a taxa de sobrevida diminui à medida que há aumento do número de linfonodos acometidos. O estadiamento III, de um modo geral, trata-se de doença localmente avançada. O prognóstico é ruim em termos de sobrevida para pacientes com metástase à distância (doença no estágio IV), mas o tratamento paliativo pode prolongar e melhorar a qualidade de vida (Greene and Sobin 2009).

Sabe-se que o *status* linfonodal é o fator prognóstico isolado mais importante do câncer de mama e que o tamanho tumoral correlaciona-se com o número de linfonodos acometidos, mas pode representar um fator prognóstico independente. Diante da doença localizada, ou seja, linfonodo negativo e ausência de metástase à distância (N0 M0), o tamanho tumoral passa a ser considerado o mais importante fator prognóstico, sendo que tumores menores apresentam maior sobrevida e menor índice de recidiva (WHO 2015). Nos últimos 40 anos, a sobrevida das pacientes com carcinoma invasivo da mama vem aumentando nos países desenvolvidos e, atualmente, é de 85% em cinco anos, enquanto, nos países em desenvolvimento, permanece com valores entre 50% e 60%. No Brasil, a sobrevida em cinco anos aumentou 9% entre os períodos de 1995 a 1999 e 2005 a 2009 (de 78% a 87%), sendo comparável aos países desenvolvidos (INCA 2016).

A patogenia do câncer de mama ainda não é totalmente elucidada, mas estudos epidemiológicos e genéticos têm descrito fatores associados a um maior risco da doença. A etiologia para seu desenvolvimento é multifatorial, sendo que alguns fatores tem maior influência, como idade, vida reprodutiva da mulher, história familiar, consumo de álcool, excesso de peso, sedentarismo, exposição à radiação ionizante e alta densidade do tecido mamário. As taxas de incidência aumentam até os 50 anos, após essa idade, o aumento ocorre de forma mais lenta, o que reforça a participação dos hormônios femininos na etiologia da doença (INCA 2016).

A história familiar também é um fator de risco bem estabelecido sendo aumentado cerca de duas a três vezes em parentes de primeiro grau de mulheres acometidas, em relação à população geral. Aproximadamente 10% dos cânceres de mama são causados por mutações nos genes, principalmente *BRCA1* (câncer de mama 1) e *BRCA2* (câncer de mama 2) e o *TP53* (*Tumor protein p53*). O *BRCA1* e *BRCA2* são os genes mais importantes para a predisposição genética ao câncer de mama (Walavalkar, Khan et al. 2015).

### **1.1.2. Subtipos do câncer de mama**

O câncer de mama é uma doença complexa, de alta heterogeneidade clínica, morfológica e biológica, com diferentes respostas terapêuticas e evoluções variáveis, justificadas pela existência de diferentes formas moleculares. Distintos perfis de expressão gênica foram caracterizados, possibilitando a identificação de subtipos moleculares de acordo com seu comportamento biológico e resposta terapêutica (Perou, Sorlie et al. 2000).

O progresso na área de biotecnologia permitiu uma classificação molecular por perfis de expressão gênica pela metodologia do *microarray cDNA*, inicialmente classificados em luminal A, luminal B, superexpressão de HER2, basalóide e *normal-like*. Posteriormente foram descritos os subtipos *claudin-low* e o molecular apócrino (Aleskandarany, Sonbul et al. 2015).

Existe uma correlação entre a classificação por *microarray cDNA* e reações imunohistoquímicas (IHQ), que determinam o perfil de expressão protéica em material de biópsia de rotina em bloco de parafina. Esta classificação molecular aproximada, é utilizada de rotina, sendo critério para definir tratamento. São usados para esse fim os seguintes biomarcadores: receptores hormonais de estrógeno (RE) e de progesterona (RP), oncogene HER2 (receptor do fator de crescimento epidérmico 2) ou amplificação do oncogene HER-2

pelo método de hibridização *in situ* por fluorescência (FISH), e o antígeno nuclear (Ki67) presente em todas as fases não G0 do ciclo celular, sendo este último um marcador útil na avaliação da proliferação celular das células neoplásicas. Além desses, são também utilizados os biomarcadores, citoqueratinas 5 e 6 (CK5/6) e receptor do fator de crescimento epidérmico 1 ou HER-1 (EGFR), os quais são expressos em células basais da mama (Cheang, Chia et al. 2009, Bhargava, Beriwal et al. 2010).

Segundo Barros and Leite (2015), os subgrupos tumorais apresentam similaridades e diferenças tanto na expressão de genes, ritmo de crescimento, vias de sinalização, composição celular, prognóstico e sensibilidade à terapia. Assim, os quatro subtipos moleculares principais na imunohistoquímica são:

1) Luminal A - tipo tumoral com origem em células epiteliais diferenciadas, apresenta expressão para RE e RP e ausência de expressão para o produto proteico do oncogene HER2. A avaliação de Ki67 evidencia baixa taxa de proliferação (<14%). Corresponde a cerca de 30 a 40% dos casos de carcinomas invasivos de mama. São tumores de bom prognóstico e bastante sensíveis à hormonioterapia.

2) Luminal B - tipo tumoral que se origina em células luminais ricas em RE. Os RP podem estar presentes tanto em alta como em baixa proporção de células. A proteína do oncogene HER2 pode ou não ser detectada e o Ki67 é mais elevado (> 14%). Corresponde a cerca de 20 a 30% dos carcinomas de mama. Tem prognóstico intermediário, sendo também sensível à hormonioterapia. Nos casos com HER2 positivos (Luminal híbrido), pode ser empregada terapia alvo anti-HER2 (anticorpo monoclonal humanizado Herceptin (trastuzumab).

3) Superexpressão do HER2 – tipo tumoral onde o oncogene HER2 está sempre superexpresso e geralmente não expressam os RE e RP. Corresponde à cerca de 15 a 20% dos casos. Geralmente este subtipo estava associado a prognóstico ruim, panorama que foi mudado com a introdução da terapia alvo, anti-HER2, pelo trastuzumab em combinação com a quimioterapia.

4) Triplo-negativos- tipo tumoral conhecido como carcinoma triplo-negativo (TN), definido pelo perfil imunohistoquímico, caracterizado pela negatividade de expressão para RE, RP e para o produto do oncogene HER2 com taxa de proliferação alta (Ki67 >14%). A maioria dos carcinomas TN são do subtipo *Basal-like* (basalóide) que compartilham certas similaridades entretanto não são sinônimos, já que a denominação basalóide é definida por expressão gênica. Esses carcinomas expressam proteínas normalmente expressas em células

basais da mama. O painel de marcadores para tumores TN tipo basal seria a ausência de expressão de RE, RP e HER-2 e expressão de CK5/6, P63, EGFR e do proto-oncogene c-KIT (CD117). Correspondem a 15-20% dos casos e sua identificação é importante porque as pacientes costumam apresentar curso clínico adverso, sendo biologicamente mais agressivos e com predileção por metástase visceral (Kurebayashi 2009, Maegawa and Tang 2010). A maioria são histologicamente do tipo TNE, de alto grau e alto índice mitótico, alguns assumem padrão de carcinoma medular. O fenótipo basalóide pode ainda estar associado a carcinomas metaplásicos, mioepiteliais, medulares típicos e atípicos, secretores e adenoide-císticos.

Pacientes com mutação herdada em *BRCA1*, quando desenvolvem carcinoma de mama, normalmente apresentam o tipo basal-*like* (Dawson, Provenzano et al. 2009). Este tipo tumoral também está associado ao gene *TP53* e estudos epidemiológicos mostram uma alta prevalência entre as mulheres mais jovens e de ascendência Africana (Carotenuto, Roma et al. 2010). Terapias personalizadas, como a terapia hormonal e a anti-HER2, não são aplicáveis ao câncer TN (Kurebayashi 2009). Apesar da falta de terapias específicas, é sensível a antraciclinas e taxanos (Maegawa and Tang 2010), mas a recidiva precoce é comum (Carotenuto, Roma et al. 2010).

Com relação aos receptores hormonais (RH), aproximadamente um milhão de mulheres são diagnosticadas com câncer de mama a cada ano e, aproximadamente 700.000, possuem RH positivos (Piccart-Gebhart 2011). Quando comparados com tumores RH negativos, apresentam forte resposta clínica ao tratamento hormonal (Early Breast Cancer Trialists' Collaborative 2005), aparência morfológica mais diferenciada (Henderson and Patek 1998), e taxas de incidência que se elevam com o envelhecimento, ao invés de diminuir após a menopausa (Yasui and Potter 1999, Anderson, Chatterjee et al. 2002).

Além disso, é conhecido que o ambiente hormonal também influencia o curso da doença (Briskin and O'Malley 2010). Os cânceres de mama classificados pela expressão de ER e/ou PR possuem diferentes características clínicas, patológicas e moleculares (Althuis, Fergenbaum et al. 2004). Estes receptores são expressos tanto no epitélio quanto no estroma mamário (Haslam and Shyamala 1981, Daniel, Silberstein et al. 1987, Haslam 1989). Postula-se que os fatores de risco associados a esta neoplasia (como consumo de pílula anticoncepcional e reposição hormonal), podem 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 (Manjer, Malina et al. 2001, Yoo, Tajima et al. 2001).

Pacientes com tumores que expressam os RH, além de apresentarem melhor resposta à hormonioterapia, também apresentam maior sobrevida, tanto livre de doença, quanto global (Wittliff 1984, Harvey, Clark et al. 1999). Embora a terapia hormonal tenha revolucionado a gestão do câncer de mama e os resultados tenham melhorado substancialmente, a estratégia ideal continua sendo um desafio significativo, demonstrando a necessidade de estudos que visem à busca por novos marcadores.

De um modo geral, a patogênese do câncer é iniciada e modulada pela interação entre as células malignas transformadas, o estroma circundante e o sistema imune inato e adaptativo. Essas interações são complexas e componentes do sistema imune atuam tanto na defesa como contribuindo para a iniciação, crescimento, invasividade e desenvolvimento de metástase (Yaquub and Aandahl 2009). A interação entre os tumores e seu microambiente é complexa e sua compreensão é de fundamental importância para o desenvolvimento de novos marcadores prognósticos e estratégias terapêuticas (Fridman, Galon et al. 2011). Importantes componentes nesta integração são as quimiocinas produzidas e secretadas pelas células ativadas do sistema imune inato ou adaptativo que podem estimular o crescimento tumoral e a progressão do câncer.

## **1.2. Quimiocinas e seus receptores**

Poucos campos de estudo da imunologia sofreram revolução tão acentuada quanto o campo das quimiocinas. As quimiocinas foram estabelecidas como citocinas quimioatraentes em 1992 após o Encontro Internacional de Imunologia em Budapest (Lindley 1993). Constituem uma grande família de mediadores inflamatórios e imunológicos e assim como as citocinas, as quimiocinas são proteínas secretórias produzidas por leucócitos e células teciduais constitutivamente ou após indução, e exercem seus efeitos localmente de forma autócrina ou parácrina. Entretanto, as quimiocinas são muito menores que as citocinas e desempenham sua atividade via receptores com sete  $\alpha$ -hélices transmembrana acoplados à proteína G, os quais são típicos para atração de leucócitos (Baggiolini 2001).

Quimiocinas são constituídas de 70 a 130 aminoácidos com quatro resíduos de cisteína conservados (Baggiolini, Dewald et al. 1994, Baggiolini, Dewald et al. 1997). Como proteínas secretórias, são sintetizadas com uma sequência guia de 20-25 aminoácidos, a qual é retirada antes de sua liberação.

Duas famílias principais, CXC e CC, também conhecidas respectivamente como  $\alpha$  e  $\beta$  quimiocinas, são distinguidas de acordo com a posição dos dois primeiros resíduos de cisteína, os quais são separados por um aminoácido variável (CXC) ou são adjacentes (CC) na região aminoterminal (Baggiolini 2001).

No entanto, há quimiocinas de outras famílias, como as XCL1/linfotaxina- $\alpha$  e XCL2/linfotaxina- $\beta$  da família C, que possui uma única cisteína e CX3CL1/fractalcina ou neurotactina, da família CX3C na qual os dois primeiros resíduos de cisteína são separados por três resíduos aminoácidos variáveis (CX3C) (Stievano et al, 2004).

Uma nomenclatura sistemática para as quimiocinas e para seus receptores tornou-se necessária com a descoberta de muitas moléculas novas. Esta classificação baseia-se no princípio estabelecido para os receptores na Conferência Gordon em citocinas quimiotáticas de 1966. Os receptores são definidos como CXC, CC, XC e CX3C, seguidos pela letra R (receptor) e um número. As quimiocinas são definidas seguindo o mesmo padrão, baseado em sua estrutura, seguidas pela letra L (ligante) e pelo número de seu gene (Pelus, Horowitz et al. 2002).

Os efeitos das quimiocinas estendem-se muito além da atração de leucócitos aos sítios de inflamação. Evidências indicam que as quimiocinas participam no desenvolvimento dos órgãos, na angiogênese, na mobilidade de células tronco, na recirculação dos leucócitos, na regulação e no desenvolvimento imunológico e hematopoiético (Sarafí, Garcia-Zepeda et al. 1997, Luster 1998, Chensue, Lukacs et al. 2001, Pelus, Horowitz et al. 2002, Barbieri, Bajetto et al. 2010).

Para cada família de quimiocinas descrita, existem receptores respectivos, acoplados à proteína G e que medeiam suas funções junto às células-alvo. A maioria dos receptores reconhece mais de uma quimiocina e muitas quimiocinas se ligam a mais de um receptor, contudo os receptores CC ligam-se somente as quimiocinas CC, e do mesmo modo os receptores CXC ligam-se somente as quimiocinas CXC. Esta restrição ligante-receptor provavelmente está relacionada às diferenças estruturais entre as quimiocinas CC e CXC, as quais têm estruturas primárias, secundárias e terciárias similares, mas quaternárias diferentes. Nas últimas décadas as quimiocinas ganharam destaque científico, uma vez que desempenham funções cruciais no desenvolvimento de diferentes doenças e processos patológicos, incluindo a inflamação, doenças autoimunes, doenças infecciosas como o HIV/AIDS, e no câncer, particularmente regulando o processo de metástase (Zlotnik 2006).

### 1.2.1. Receptor CXCR4: implicações na tumorigênese

O CXCL12 é um ligante específico para CXCR4. Este receptor é expresso em uma grande variedade de tecidos, como tecido linfóide e nervoso central, pode mediar a migração de leucócitos e progenitores hematopoiéticos em resposta à quimiocina CXCL12. No sistema imune, é altamente expresso em monócitos, células B e células T virgens no sangue periférico, bem como em células progenitoras hematopoiéticas na medula óssea (MO) (Zou, Kottmann et al. 1998).

A interação CXCR4/CXCL12 participa da modulação da resposta imune, induzindo a apoptose de células T CD8<sup>+</sup> mediada por macrófagos e também do desenvolvimento embrionário (Koizumi, Hojo et al. 2007). Está envolvida na patofisiologia de várias doenças. Esta interação pode aumentar a sobrevivência de células-tronco malignas, propiciando sua proliferação, estando portanto envolvida nos mecanismos de invasão e de metástase, levando a disseminação de diversos tipos de cânceres, incluindo o de próstata, tumores cerebrais, e leucemias (Balkwill 2004, Kakinuma and Hwang 2006).

Assim, vias de sinalização ativadas pelo eixo CXCL12/CXCR4 podem conferir uma vantagem de crescimento às células tumorais tanto em sítios primários como em sítios metastáticos. O fator nuclear kappa B (NF-κB) ativado por sinal extracelular regula a expressão de metaloproteinases, o ativador do plasminogênio tipo uroquinases, e quimiocinas como o CXCL12 e seu receptor CXCR4 em células do câncer de mama altamente metastáticas. O NF-κB regula a motilidade e proliferação das células tumorais do câncer de mama por promover diretamente aumento da expressão do CXCR4, favorecendo a metástase (Helbig, Christopherson et al. 2003).

Dentro deste contexto, receptores de quimiocinas desempenham importantes papéis 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. Portanto, é sugerido que após crescimento inicial de tumores primários, mediados pelos receptores de quimiocinas, células malignas invadem os vasos sanguíneos, migrando então para tecidos distantes (Kakinuma and Hwang 2006). O receptor CXCR4 vem sendo implicado na disseminação de tumores malignos a partir do sítio primário, sendo visto como um marcador em vários tipos de neoplasia (Epstein 2004). Como exemplo, a expressão acentuada deste receptor tem sido associada ao desenvolvimento de metástase em câncer oral (Taki,

Higashikawa et al. 2008) e também em células do câncer de mama e está associado com prognóstico desfavorável (Miller *et al*,2001; Chu *et al*, 2010).

O gene que codifica o CXCR4 está localizando no cromossomo 2q2 e um polimorfismo de nucleotídeo único (SNP) com uma troca de base única de citosina (C) para timina (T), foi mapeado no códon 138 da proteína. Teng et al (2009) investigaram este polimorfismo genético e observaram que a presença de pelo menos um alelo mutado estaria associado com o desenvolvimento dos estadiamentos III e IV e comprometimento linfonodal em carcinomas orais. Já em outro estudo, embora Cacina, Bulgurcuoglu-Kuran et al. (2012) não tenham verificado associação significativa para o polimorfismo de CXCR4, os portadores do alelo T foram mais comuns nos pacientes comparados com os controles. Além disso, indivíduos com genótipo homozigoto TT apresentaram 2,5 vezes mais risco para carcinoma endometrial.

Com a experiência do nosso laboratório em polimorfismos genéticos humanos e expressão gênica de quimiocinas e citocinas na regulação do sistema imune e de várias doenças (do Val Carneiro, Nixdorf et al. 2009, Amarante and Watanabe 2010, Watanabe, Oda et al. 2010, Aoki, Amarante et al. 2011, Kallaur, Kaimen-Maciel et al. 2011, Magnani, Castro-Gomez et al. 2011, Watanabe, Nunes et al. 2011, Amarante, Watanabe et al. 2012, de Lima, Guembarovski et al. 2012, de Oliveira, Guembarovski et al. 2012), o presente estudo teve como proposta avaliar o polimorfismo genético no receptor CXCR4, bem como sua expressão gênica e proteica, e suas possíveis implicações na patogênese do câncer de mama.

### **1.2.2. Receptor CXCR4: implicações no câncer de mama**

O câncer de mama é caracterizado por um padrão metastático distinto, envolvendo linfonodos, medula óssea, fígado e pulmão. A migração das células tumorais metastáticas tem muita semelhança com o tráfego de leucócitos. Tem sido relatado que estas podem ser guiadas através da circulação sanguínea até órgãos distantes que expressam quimiocinas específicas como CXCL12, pois estas células possuem receptores específicos que possibilitam este evento, como o CXCR4. Desta forma, as células tumorais invadem estes órgãos possibilitando a origem de tumores secundários (Muller, Homey et al. 2001).

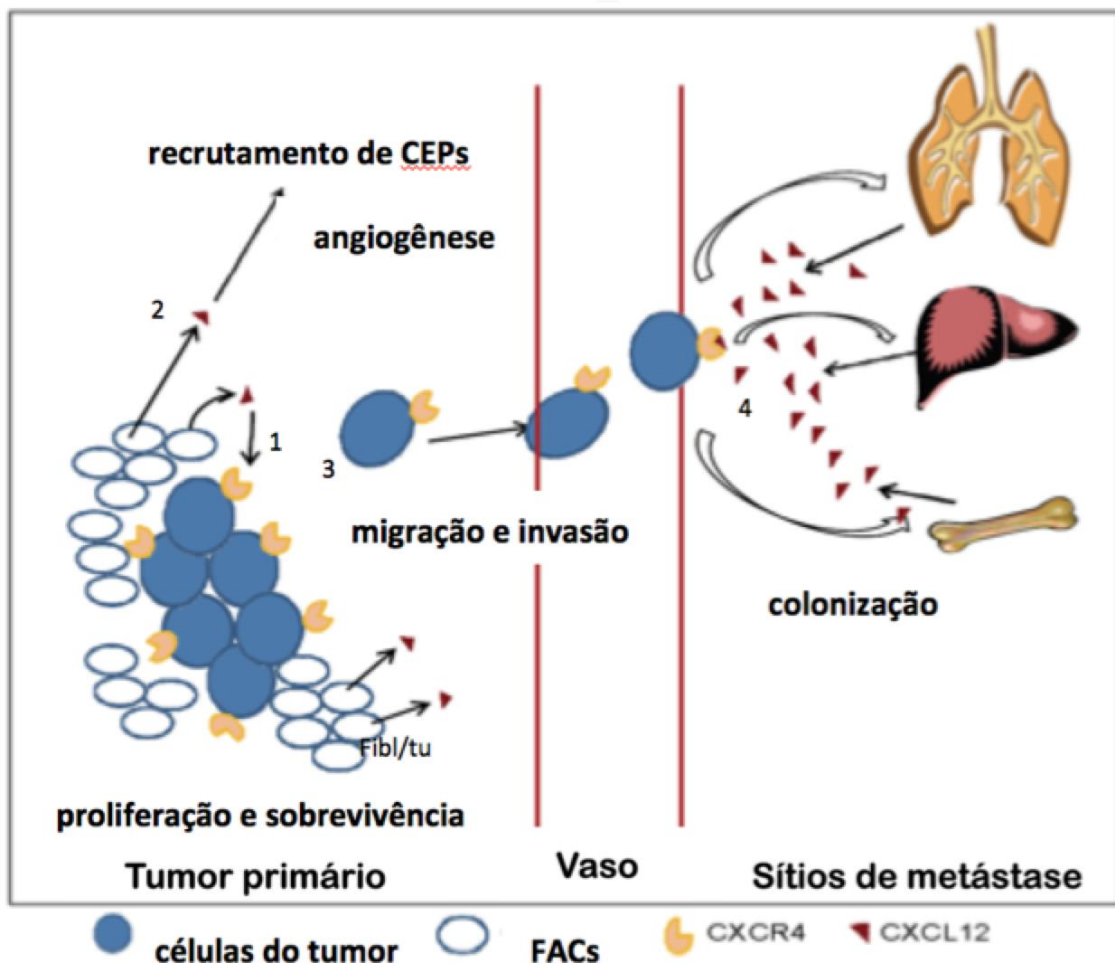
Assim, a expressão aumentada de CXCR4 tem sido descrita como um marcador prognóstico em vários tipos de câncer, incluindo o de mama (Burger and Kipps 2006). Por meio da análise de sua expressão em cortes de tecido glandular mamário normal e tumoral por imunohistoquímica, foi observado aumento de expressão de CXCR4 em células neoplásicas,

ao passo que em células normais não houve expressão deste receptor (Muller, Homey et al. 2001).

A sobrevida de pacientes com câncer de mama está associada ao processo metastático o qual diminui muito, de 90% para 20%, quando estas ocorrem. O mecanismo preciso que leva a este processo permanece pouco compreendido, mas sabe-se que este pode ser o resultado várias etapas sequenciais que se iniciam quando as células do tumor primário apresentam capacidade de migração, sobrevivência, colonizando sítios secundários com proliferação celular e a formação macrometástases (Mukherjee and Zhao 2013). Neste contexto, tem sido investigada a importância do CXCR4 na patogênese do câncer de mama (do Val Carneiro, Nixdorf et al. 2009, Oda, de Oliveira et al. 2012) e muito tem sido discutido com relação ao seu possível papel no processo metastático.

Segundo Mukherjee e Zhao, 2013, o CXCR4, juntamente com seu ligante CXCL12 estariam envolvidos nas várias etapas do processo metastático, iniciando pela ação do CXCL12 secretado pelos fibroblastos associados às células tumorais (FACs) que se liga ao CXCR4 expresso na superfície das células tumorais da mama, levando à ativação de vias proliferativas intracelulares que aumentam o crescimento tumoral. O CXCL12 recruta células endoteliais progenitoras (CEPs) da medula óssea para o tumor, aumentando a angiogênese e contribuindo para o crescimento tumoral.

A ligação CXCL12/CXCR4 leva a migração de células tumorais CXCR4+ para além do tumor primário em resposta a um gradiente de concentração do CXCL12. Nesta etapa do processo metastático as células adquirem capacidade de motilidade e de invasividade.



**Figura 1. Eixo CXCL12/CXCR4 na migração de células tumorais.**

**Fonte:** Mukherjee e Zhao, 2013.

Tem sido hipotetizado que estas moléculas estão associadas com a transição epitelial-mesenquimal (EMT), um processo pelo qual as células epiteliais adquirem propriedades mesenquimal fibroblastos-like e apresentam reduzida adesão intercelular e aumento da capacidade de motilidade e invasão do estroma e vascular. O CXCL12 funciona como quimioatraente que guia células do tumor primário para órgãos ricos em CXCL12, levando ao desenvolvimento de metástases (Tam.W.L. e Weinberg R.A. 2013).

Além disso, células de câncer de mama expressam níveis elevados de CXCR4, os quais podem direcionar a quimiotaxia e podem conduzir a ETM juntamente com uma regulação positiva de outros receptores de quimiocinas e citocinas, levando à migração celular, invasão linfática e metástase tumoral (Muller et al., 2001, Mukherjee e Zhao 2013), e células metastáticas mostram uma forte expressão deste receptor, sendo este aumento associado a uma baixa sobrevida das pacientes (Luker and Luker 2006).

Holland, Kochetkova et al. (2006) analisaram a expressão de CXCR4 em linhagens celulares a fim de investigar sua relação com a capacidade invasiva das células transformadas, e sugeriram que a expressão deste receptor estaria correlacionada com o potencial invasivo, sendo, portanto, um marcador adequado para prever metástase.

Além disso, pequenas moléculas ou peptídeos inibitórios de receptores de quimiocinas tendo CXCR4 como alvo têm apresentado efeitos sobre o crescimento tumoral em modelos animais. A utilização de antagonistas de CXCR4 no tratamento de tumores metastáticos baseia-se no fato de haver um aumento na expressão desse receptor pelas células tumorais. A inibição destes receptores poderia deixar as células tumorais mais vulneráveis aos tratamentos antitumorais (Lee, Kakinuma et al. 2006).

Assim sendo, análises que envolvam marcadores de respostas imunes efetoras no câncer de mama são de fundamental importância para a elaboração de estratégias de intervenção imune efetivas. Sabe-se que as metástases representam um risco fatal na evolução dos tumores da mama. Diante desse quadro, novas pesquisas apontam no sentido de evitar esses mecanismos, tentando, por exemplo, interferir na interação entre receptores e suas quimiocinas específicas. Portanto, essas moléculas podem ser alvos terapêuticos interessantes, o que reforça a importância de estudos envolvendo o receptor CXCR4 dentro do contexto da carcinogênese mamária.

### **1.3. Gene *TP53* e correlação com o eixo *CXCL12/CXCR4***

A origem e o desenvolvimento neoplásico são caracterizados por alterações em dois grupos principais de genes: os proto-oncogenes e os genes supressores tumorais (Hanahan and Weinberg 2000). Os genes supressores tumorais atuam como reguladores negativos da proliferação (Verma and Triantafillou 1998) uma vez que as proteínas codificadas por eles fazem parte das vias de sinalização celular e atuam retardando a progressão do ciclo celular, bloqueando a diferenciação ou induzindo a morte programada. Assim, mutações que levam a inativação destes genes liberam a célula da inibição imposta pelos mesmos, levando à proliferação desordenada (Weinberg 1991).

O gene *TP53* é um supressor tumoral que age na regulação do desenvolvimento e do crescimento celular. Está localizado no braço curto do cromossomo 17 (17p13.1) e codifica uma fosfoproteína de 53kDa a qual desempenha um papel importante no controle do ciclo celular, induzindo reparo de DNA ou apoptose (Eisenberg 2001, Einsiedel, von Stackelberg et al. 2005, Almeida 2007). Este gene é ativado em resposta a sinais de dano e interage com,

pelo menos, 6 outros genes (Xiong, Hannon et al. 1993). De um modo geral, a ativação da proteína p53 resulta na indução ou repressão de um grande número de genes envolvidos no reparo de DNA e na morte celular, protegendo, assim, contra o acúmulo de alterações genéticas (Rossner, Gammon et al. 2009).

Polimorfismos genéticos foram descritos neste gene, e o mais informativo deles está localizado no éxon 4, códon 72, e tem sido investigado em relação a várias neoplasias humanas (Kietthubthew, Sriplung et al. 2003). Este polimorfismo ocorre por simples substituição de uma base G para C, com alteração estrutural da proteína (Thomas, Kalita et al. 1999). O códon 72 codifica o aminoácido arginina (CGC; Arg72) ou o prolina (CCC; Pro72), correspondendo ao fenótipo arginina/prolina (Arg/Pro) (Tada, Furuuchi et al. 2001) com distintas características bioquímicas e biológicas. Consequentemente, três genótipos diferentes são criados, homozigoto GG (Arg/Arg), homozigoto CC (Pro/Pro) e heterozigoto GC (Arg/Pro).

Pesquisas mostram que o genótipo homozigoto GG (Arg/Arg) poderia estar relacionado com risco aumentado para o desenvolvimento tumoral. Estudo realizado por Aoki, da Silva do Amaral Herrera et al. (2009), em pacientes com câncer de mama, encontrou associação positiva para o genótipo homozigoto GG.

Siddique and Sabapathy (2006), demonstraram que o alelo que codifica para o aminoácido prolina é mais eficaz quanto à capacidade de reparação do DNA. Já Bergamaschi, Samuels et al. (2006), relatam que o alelo que codifica para o aminoácido arginina aumenta a capacidade da proteína p53 em induzir a apoptose, e aquele que codifica para prolina aumenta o bloqueio na fase G1 do ciclo celular, possibilitando um reparo mais eficaz dos danos no DNA. Assim, é importante observar que ambos os alelos, que codificam para os aminoácidos arginina ou prolina, são dotados de propriedades de apoptose e reparo, mas com diferentes eficiências.

Os genes *CXCL12* e *TP53* são descritos como envolvidos na migração de células metastáticas em alguns tipos de câncer (Mehta, Christopherson et al. 2007, Yeudall, Vaughan et al. 2012, Panka, Liu et al. 2013). Segundo Moskovits, Kalinkovich et al. (2006), em estudo envolvendo cultura de fibroblastos (de camundongo e humano), a proteína p53 pode suprimir a produção de CXCL12, enquanto que a supressão do gene *TP53* nas células em cultura leva ao aumento da secreção de CXCL12, aumentando, por sua vez, a migração e a invasão das células tumorais.

Dentro deste contexto, o polimorfismo no códon 72 do gene *TP53* pode modificar a função da proteína final e influenciar na suscetibilidade ao câncer. Dada a interação entre as

proteínas p53 e o eixo CXCL12/CXCR4, estes marcadores constituem candidatos a serem avaliados quando se busca novos marcadores para a neoplasia da mama.

## 2. OBJETIVOS

### 2.1. Objetivo geral

Analisar aspectos moleculares (polimorfismo genético e expressão gênica) e histopatológicos (expressão proteica) do receptor de quimiocina CXCR4, bem como uma possível correlação com o polimorfismo (rs1042522) no gene *TP53*, e avaliar possíveis implicações na patogênese do câncer de mama, em uma amostra geral e em uma amostra luminal/HER2(-).

### 2.2. Objetivos específicos

#### Artigo experimental 1

- ❖ Analisar o polimorfismo (rs2228014) no gene *CXCR4* em pacientes com câncer de mama e procurar uma correlação com parâmetros prognósticos da doença;
- ❖ Avaliar a expressão gênica de *CXCR4* em tecido tumoral e normal da mama;
- ❖ Avaliar a expressão da proteína CXCR4 em amostras de tecidos tumorais e normais adjacentes da mama;
- ❖ Avaliar a presença do polimorfismo em relação às expressões gênica e proteica de CXCR4;
- ❖ Correlacionar os resultados de expressão gênica e proteica de CXCR4.

#### Artigo experimental 2

- ❖ Avaliar o polimorfismo (rs1042522) no gene *TP53* e correlacionar com dados prognósticos de pacientes de uma amostra de câncer de mama luminal/HER2(-);
- ❖ Avaliar a expressão gênica de CXCR4 e correlacionar com dados prognósticos de pacientes de uma amostra com câncer de mama luminal/HER2(-);
- ❖ Avaliar a associação dos diferentes genótipos do polimorfismo (rs1042522) do *TP53* com a expressão gênica de CXCR4 em pacientes com câncer de mama luminal/HER2(-).

### **3. MATERIAIS E MÉTODOS**

#### **3.1. Aspectos éticos**

Este projeto foi aprovado pelo Comitê de Ética em Pesquisa Envolvendo Seres Humanos – Universidade Estadual de Londrina, 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 (Anexos).

#### **3.2. Seleção e caracterização das amostras**

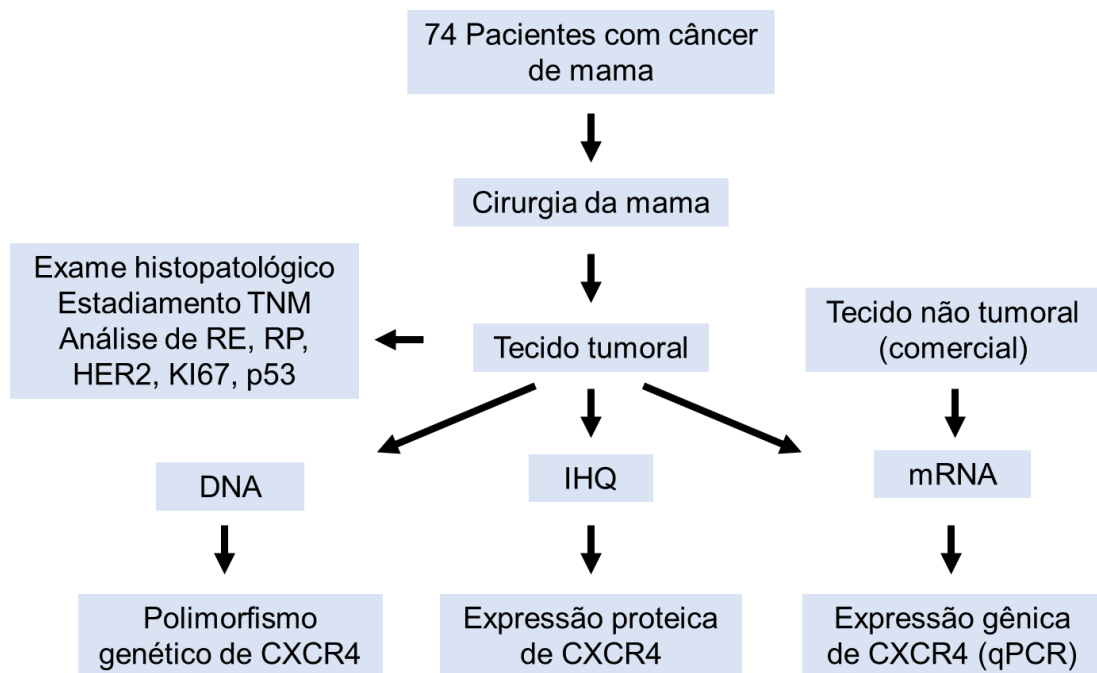
A amostra do primeiro artigo experimental foi constituída por 74 tecidos mamários obtidos por ressecção cirúrgica de pacientes que tiveram diagnóstico confirmado de neoplasia mamária, e as quais eram livres de quimioterapia neoadjuvante e/ou adjuvante. A amostra do segundo artigo experimental foi constituída por 33 pacientes livres de quimioterapia neoadjuvante e/ou adjuvante, sendo esta luminal/HER2(-). A amostra controle foi constituída por 146 mulheres livres de neoplasia mamária, confirmada por exame clínico e mamografia, e sem história familiar de câncer de mama.

O convite às pacientes para participarem deste projeto de pesquisa ocorreu durante o seu atendimento clínico no Hospital do Câncer de Londrina (Londrina, Paraná, Brasil). As atividades de pesquisa foram realizadas no Laboratório de Polimorfismos de DNA, Imunologia e Genética Molecular, Departamento de Ciências Patológicas, Centro de Ciências Biológicas da Universidade Estadual de Londrina.

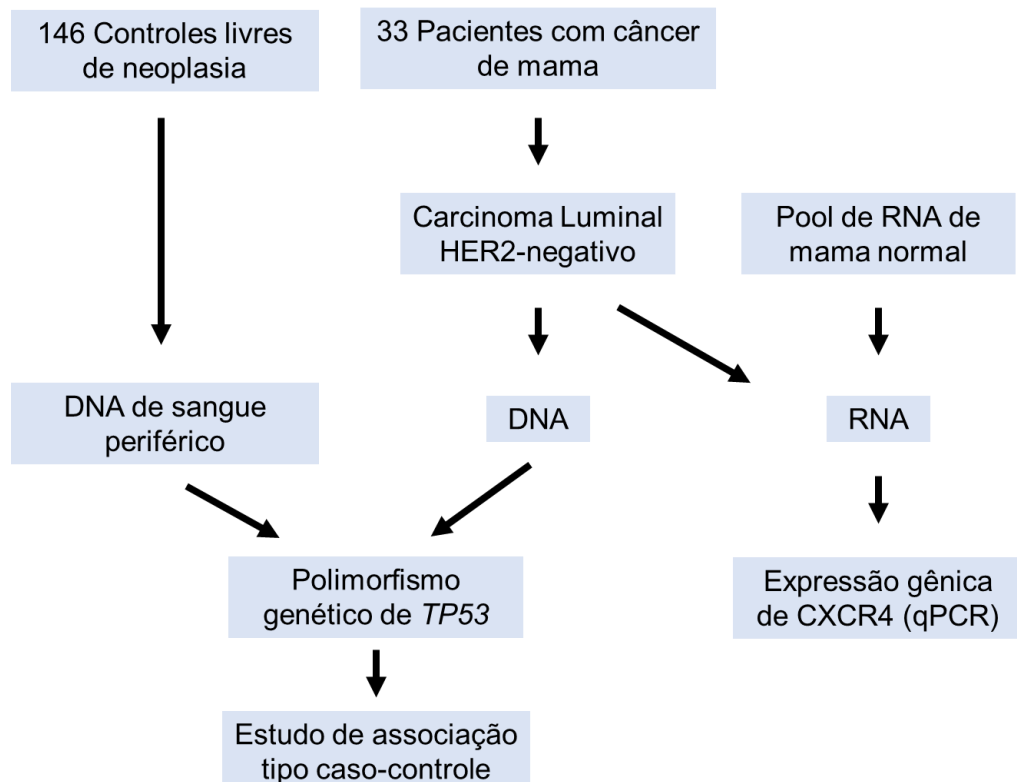
#### **3.2. Estadiamento do câncer de mama segundo UICC**

A classificação dos tumores utilizada no presente estudo seguiu a sistematização da UICC, denominada TNM. Após a avaliação destes fatores, os casos foram classificados em estadiamentos que variaram de I a IV graus crescentes de gravidade da doença (INCA/MS/2007), os quais 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).

### Delineamento experimental do primeiro artigo



### Delineamento experimental do segundo artigo



### 3.3. Obtenção de DNA e RNA a partir do tecido mamário

Os tecidos mamários tumoral e normal pós-cirúrgico, previamente separados pelo anatomopatologista, foram homogeneizados por pinçamentos sucessivos em solução fisiológica estéril e, posteriormente, centrifugados a 2000 rpm para obtenção das células.

A partir destas foram obtidos DNA e RNA, tanto dos tecidos normais quanto dos tumorais, para a realização das análises propostas.

Os DNAs foram obtidos após digestão proteica com Proteinase K pelo método de *Salting out* (Kirby 1993). Os RNAs foram obtidos após adição de 500 µL de TRIzol-LS (Invitrogen, Carlsbad, USA), segundo as instruções do fabricante.

Todas as amostras obtidas foram quantificadas por espectrofotometria a 260 nm em espectrofotômetro (NanoDrop 2000®, NanoDrop Technologies, Wilmington, DE, EUA). O grau de pureza em relação à contaminação por proteínas foi avaliado pela razão entre as absorvâncias nos comprimentos de 260 nm e 280 nm.

### 3.4. Reação em Cadeia da Polimerase (PCR)

O método de PCR foi utilizado para a detecção da variante polimórfica do gene *CXCR4* em 74 amostras de pacientes com câncer de mama e para a detecção da variante do gene *TP53* em 33 amostras de pacientes luminal/HER2(-), e seus respectivos controles livres de neoplasia mamária.

Todas as genotipagens foram realizadas no Laboratório de Polimorfismos de DNA e Imunologia da Universidade Estadual de Londrina, na presença de controles negativos para avaliação de contaminação dos reagentes e marcadores de tamanho de fragmento, para confirmação dos tamanhos dos produtos de PCR.

#### 3.4.1. Análise do polimorfismo rs2228014 do gene *CXCR4*

Para a determinação do polimorfismo do *CXCR4*, foram utilizados protocolos descritos previamente (Teng, Liu et al. 2009), com os oligonucleotídeos iniciadores: *Forward*: 5' - AACTTCCTATGCAAGGCAGT - 3' e *Reverse*: 5' TATCTGTCATCTGCCTCACT - 3', seguindo as etapas de ciclagem descritas na Tabela 1.

Após a amplificação, foi realizada uma digestão enzimática utilizando-se 2U da endonuclease de restrição *BclI* (New England Biolabs, New England, UK) e 10 µL do produto de PCR. Os tubos foram incubados por 4 horas a 37°C e os resultados foram observados pela clivagem ou não do fragmento original de 236 pb.

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



Quando o indivíduo possui uma troca de nucleotídeo C por T, a enzima não reconhece seu sítio de ligação e o fragmento permanece intacto (236pb). Sendo assim, o indivíduo homocigoto prevalente sofre clivagem do fragmento de 236 pb em 2 fragmentos um contendo 133 pb e outro de 103 pb. O indivíduo heterocigoto apresenta 3 fragmentos: 236 pb, 133 pb e 103 pb. O indivíduo que possui o polimorfismo em homocigose (genótipo raro) apresenta um fragmento de 236 pb, o qual não sofre ação da enzima de restrição.

**Tabela 1.** Protocolo de ciclagem da reação em cadeia da polimerase (PCR) para a genotipagem do polimorfismo rs2228014 do gene *CXCR4*.

<b>rs2228014 (<i>CXCR4</i>)</b>			
	<b>T °C</b>	<b>Tempo</b>	<b>Ciclos</b>
Estágio 1	94°C	5min	1 ciclo
Estágio 2	94°C	45s	35 ciclos
	60°C	1min	
	72°C	75s	
Estágio 3	72°C	10min	1 ciclo

T °C: temperatura

Os produtos de PCR, bem como os produtos de clivagem, foram submetidos à eletroforese em gel de poliacrilamida 10%, utilizando-se o tampão de carregamento Tris-Acetato-EDTA (TAE) numa voltagem de 100 V por cerca de 2 horas. Utilizou-se 3 µL de xileno cianol (XC), 10 µL de cada amostra e 1 µL do marcador de tamanho de fragmento de 100 pb (*Ladder* 100 bp, Invitrogen, Carlsbad, CA, USA).

### 3.4.2. Análise do polimorfismo rs1042522 do códon 72 do gene *TP53*

O DNA foi amplificado por PCR com iniciadores específicos, descritos em estudos anteriores (Papadakis, Soultzis et al., 2002, Lima, Serafim et al., 2006, Aoki, da Silva do Amaral Herrera et al., 2009), segundo o número de acesso do *GenBank*: AY838896 (Tabela 2).

Cada amostra de DNA foi amplificada duas vezes, com iniciadores para os alelos contendo G (Arginina) ou C (Prolina), sempre utilizando uma amostra de genótipo heterozigoto como controle de amplificação.

Os produtos de PCR foram analisados por eletroforese em gel de poliacrilamida (10%) e detectados por coloração com nitrato de prata. O alelo C (Prolina) produz um fragmento de 178 pb, enquanto que o alelo G (Arginina) produz um fragmento de 136 pb. O indivíduo que apresenta o genótipo heterozigoto para este polimorfismo apresenta ambos os tamanhos de fragmento (136/178 pb).

**Tabela 2.** Sequencia dos *primers* e produtos de PCR para o polimorfismo do gene *TP53*.

Gene	Sequência dos <i>primers</i>	Produto de PCR
<i>TP53</i> (rs1042522)	(alelo G) Arg	
	5' TCC CCC TTG CCG TCC CAA 3'	136 bp (Arg)
	5' CTG GTG CAG GGG CCA CGC 3'	
	(alelo C) Pro	
5' GCC AGA GGC TGC TCC CCC 3'	178 (Pro)	
5' CGT GCA AGT CAC AGA CTT 3'		

### 3.5. Reação em Cadeia da Polimerase em tempo real, quantitativo (qPCR)

A síntese de DNA complementar foi realizada a partir de 500 ng de RNA, 20 U de transcriptase reversa *Moloney Murine Leukemia Virus Reverse Transcriptase* (M-MLV RT; Invitrogen™), 4 U de Inibidor de Ribonuclease Recombinante (RNase OUT™; Invitrogen™), sob as seguintes condições: 0,4 µM oligo dT, 40 mM TrisHCl (pH 8.3), 60 mM KCl, 6,4 mM MgCl<sub>2</sub>, 0,2 mM de dNTP, a 37°C por 60 min no termociclador Hybaid PCR Sprint Thermal Cycler.

Como gene constitutivo foi utilizado o *GAPDH* (gliceraldeído-3-fosfato desidrogenase). As reações foram realizadas em duplicata para cada amostra de DNA complementar (DNAc) utilizada. A quantidade de DNAc utilizada foi previamente determinada através de uma curva de amplificação do gene normalizador *GAPDH* na qual foram utilizadas diferentes diluições de DNAc (1:5, 1:10, 1:20; 1:40 e 1:80). Para a realização da curva, foi utilizada uma amostra aleatória de DNAc preparada em duplicata.

A reação de qPCR foi realizada a partir do DNAc, utilizando o fluoróforo Platinum®SYBR Green qPCR SuperMix UDG (Invitrogen™) e 0.25 µM dos iniciadores descritos na tabela 3. 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

**Tabela 3.** Condições da Reação de PCR Quantitativa

Gene alvo	Nº de acesso GenBank	Iniciador	Sequência	Temperatura de Anelamento
<i>CXCR4</i>	AF025375	<i>Foward</i>	5' TGTTGGCTGAAAAGGTGGTC 3'	54°C
		<i>Reverse</i>	5' AAAGATGAAGTCGGGAATAGTC 3'	
<i>GAPDH</i>	NM_002046	<i>Foward</i>	5' GAA GGT GAA GGT CGG A 3'	54°C
		<i>Reverse</i>	5' GGG TCA TTG ATG GCA AC 3'	

As sequências dos iniciadores para o *GAPDH* foram obtidas do Hospital do Câncer A.C. Camargo de São Paulo, o qual é utilizado rotineiramente para PCR quantitativo nas análises de expressão gênica em células humanas. As sequências dos *primers* para *CXCR4* foram obtidas de acordo com a análise no Software *GeneRunner* (Hasting Software Inc., Nova Iorque, EUA).

A análise da curva de *melting* revelou a presença de somente um pico correspondente aos fragmentos do *GAPDH* ou do *CXCR4* - na temperatura de 80,5°C, confirmando a especificidade da amplificação.

### 3.6. Cálculo para expressão de RNAm

A expressão relativa de *CXCR4* foi calculada pelo método do  $2^{-\Delta\Delta CT}$  e normalizada de acordo com o gene constitutivo *GAPDH*. Um *pool* de RNA comercial (Clontech Laboratories Inc., Mountain View, CA, USA) foi utilizado como controle.

### 3.7. Análise da expressão de CXCR4 por IHQ

Amostras de tecido normal e tumoral fixadas em formalina e embebidas em parafina foram cortadas com uma espessura de 4  $\mu$ m, as quais foram fixadas em lâmina silanizada (Starfrost, Knittel, Alemanha). As amostras foram aquecidas a 56°C, desparafinizadas em xilol e reidratadas em álcool. A recuperação antigênica foi realizada utilizando tampão de ácido cítrico (1,8 mM) e citrato de sódio di-hidratado (8,2 mM) para posterior adição do anticorpo primário.

O anticorpo primário utilizado para detecção do receptor *CXCR4* foi um anticorpo de camundongo anti-*CXCR4* (CD184) humano purificado (clone *polyclonal*; eBioscience, San Diego, CA, EUA), em uma diluição de 1:100, previamente estabelecida após testes com diferentes diluições (1:800; 1:600; 1:400; 1:200; 1:100), até obter-se um padrão de imunocoloração limpo, sem *background* e que se assemelhasse aos observados na literatura (Yanan, Sun et al. 2014).

O anticorpo secundário (*Mouse/Rabbit Immuno detector HRP/DAB*, Bio SB INC, Santa Barbara, CA, USA) foi adicionado, as lâminas foram contra coradas com hematoxilina de Gill e a lamínula foi fixada utilizando-se bálsamo do Canadá.

Foram realizados controles de especificidade do anticorpo primário, em amostras positivas e sem a adição do mesmo. A marcação para *CXCR4* foi avaliada no tecido tumoral e normal adjacente. A leitura foi realizada em microscópio óptico (Eclipse-E200, Nikon, Japão), por dois patologistas especializados e de forma semi-quantitativa. O protocolo para análise deste marcador foi estabelecido no próprio Laboratório de Pesquisa. Para a marcação em tecido tumoral e normal adjacente considerou-se: 0= sem imunomarcação; += imunomarcação leve; ++= imunomarcação moderada; +++= imunomarcação forte.

### **3.8. Análise Estatística**

Os testes do Qui-Quadrado de Pearson e as Correlações de Spearman Rho ou Tau-b de Kendall foram utilizados na comparação entre os resultados de expressão gênica, expressão proteica e polimorfismo genético de *CXCR4*, bem como em relação ao polimorfismo do gene *TP53*, e em relação aos parâmetros prognósticos das pacientes, pelo programa estatístico SPSS versão 22.0 (SPSS Inc., Chicago, Illinois, USA). Valores de  $p < 0,05$  foram considerados significativos.

O estudo de associação do tipo caso-controle para o polimorfismo do gene *TP53* foi realizado pelo cálculo da *Odds Ratio* (OR) com Intervalo de Confiança a 95% (IC=95%).

## 4. PRODUÇÃO CIENTÍFICA

### 4.1. ARTIGO 1

Hindawi Publishing Corporation  
Analytical Cellular Pathology  
Volume 2015, Article ID 891020, 6 pages  
<http://dx.doi.org/10.1155/2015/891020>

#### *Review Article*

## **Immunohistochemical Expression of CXCR4 on Breast Cancer and Its Clinical Significance**

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Received 30 April 2015; Revised 3 June 2015; Accepted 8 June 2015

Academic Editor: Elena Adinolfi

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Many tumor cells express chemokines and chemokine receptors, and, for this reason, these molecules can affect the tumor progression. It is known that breast cancer is a complex and heterogeneous neoplasia comprising distinct diseases, histological characteristics, and clinical outcomes. The most studied role for CXCL12 chemokine and its receptor CXCR4 in breast cancer pathogenesis is the metastasis event, although several reports have demonstrated its involvement in other processes, such as angiogenesis and tumor growth. It has been found that CXCR4 is required for breast cancer cell migration to other sites such as lung, bone, and lymph nodes, which express high levels of CXCL12 chemokine. Therefore, CXCR4 is being considered a prognostic marker in breast cancer. Within this context, this review summarizes established studies involving expression of CXCR4 on breast cancer, focusing on its clinical significance.

### **1. Introduction**

Chemokines are potent chemotactic molecules involved not only in inflammation processes and immune surveillance but also in important physiological and pathological conditions, such as cancer progression [1]. Some data have suggested that the chemokine receptor network could be involved in the seed and soil phenomenon and that metastasis could be localized according to chemokine receptor expression [2].

Many retrospective studies have documented the expression of various chemokine receptors, particularly the C-X-C chemokine receptor 4 (CXCR4), and the association with higher recurrence incidences and cancer-related deaths, tumor size, advanced tumor-node-metastasis (TNM) stage, and shorter survival, predicting poor prognosis to cancer patients [3, 4].

Among different types of malignant tumours, breast cancer is the most common among women, in both developed and developing countries, with approximately 1,7 million new cases and 560,000 deaths worldwide every year [5, 6]. During the last decade, researches have focused deeply on the molecular biology of this disease. Technological breakthroughs and in particular high throughput approaches have allowed researchers to inquire into the nature of breast cancer, uncovering intrinsic and orchestrated interconnection of several signaling pathways, and the cellular microenvironment influencing disease pathophysiology, outcome, and treatment response [7].

There are opposing reports in the literature regarding the expression of CXCR4 in breast cancer stating that it is increased, decreased, or not changed compared to benign epithelium. Furthermore, there is disagreement amongst the

authors with regard to CXCR4 localization in malignant cells: some describe peripheral or nuclear staining, while the majority states that CXCR4 expression is predominantly cytoplasmic.

The tumoral CXCR4 expression has been evaluated by several methods, one of which being commonly used is the standard immunohistochemical staining. Published data demonstrated the analysis of CXCR4 expression by immunohistochemistry in breast cancer tissue and cells and its clinical significance regarding the localization and expression patterns, emphasizing its distinguished relevance as a prognostic marker. Inside this context, this review focused on reporting the importance of immunohistochemical expression of CXCR4 on breast cancer and its clinical significance.

## 2. CXCR4 and Breast Cancer

CXCR4 is a rhodopsin-like G-protein coupled receptor (GPCR), displaying 7-transmembrane helical domains, involved in important signaling functions. The GPCRs signaling pathway has been discussed in a review by Cojoc et al. [8]. Briefly, upon ligand interaction and receptor activation, the GPCR promotes conformation changes within the trimeric G protein ( $G\alpha/G\beta/G\gamma$ ), releasing from the receptor the  $G\alpha$  and  $G\beta\gamma$  subunits, which interact with various effector proteins and initiate intracellular signaling cascades, leading to several processes, such as stemness, survival, proliferation, and chemotaxis.

The CXCL12 chemokine and its receptor CXCR4 are molecules with important immunological functions, which have further relevant roles in the context of mammary carcinogenesis. Thus, the significance of the CXCL12/CXCR4 axis in breast cancer invasion and metastasis has been widely investigated [9–11].

CC and CXC chemokine receptor expression is described on a wide variety of cancer cell types [12–14], including at least 23 different hematopoietic, epithelial, and mesenchymal tumours [12]. On the other hand, within primary tumours and cancer cell lines, for example, ovarian and non-small-cell lung cancer, only a subpopulation of cells expresses the CXCR4 receptor [15].

In human cells, CXCR4 expression was detected in many cells, for instance, bronchial epithelial cells [16], endothelial cells [17, 18], fibrocytes [19], lymphocytes [20], intestinal (including colonic) epithelial cells [21, 22], microglia, neurons, and astrocytes [23], primitive hematopoietic progenitor cells [24], vascular smooth muscle cells [25], and pluripotent stem cells, including mammary stem cells [26]. Further, according to Müller et al. [27], CXCR4 is present at a low level or even absent in normal breast tissue but is highly expressed in both primary and metastatic breast tumours.

Particularly, CXCR4 expression on breast tumor cells can be regulated by several factors, such as hypoxia, vascular endothelial growth factor (VEGF), nuclear factor kappa-light-chain-enhancer of activated B cells (NF- $\kappa$ B), estrogen, transforming growth factor-beta 1 (TGF- $\beta$ 1), and IFN- $\gamma$ , which have been shown to upregulate this receptor in tumor microenvironment [28–30]. Furthermore, its expression can

be regulated by epigenetic mechanisms, like cytosine methylation, whose pattern was correlated with clinicopathological aspects of primary breast tumours, in a study developed by Ramos et al. [31].

As peripheral lymphocytes preferentially localize to peripheral lymphoid tissues, such as lymph nodes, chemokine receptors in tumoral cells drive invasion and metastatization to specific sites. Indeed, CXCR4 is overexpressed in metastatic breast cancer cells; consequently it critically mediates the homing process to specific metastatic sites [27]. Notably, this is the most devastating process attributed to cancer and significantly influences its morbidity and mortality [32].

The first steps of metastasis are characterized by increased motility and invasiveness, and it has been hypothesized that they are associated with the epithelial-mesenchymal transition (EMT), a process by which epithelial cells acquire mesenchymal fibroblast-like properties and show reduced intercellular adhesion and increased motility [33]. Notwithstanding the CXCR4 involvement in chemotaxis process, CXCR4 can drive epithelial to mesenchymal transition along with an upregulation of other chemokine receptors and cytokines, leading to cell migration, lymphatic invasion, and tumor metastasis [34].

## 3. Immunohistochemical Expression of CXCR4 on Breast Cancer

Application of immunohistochemistry in clinical laboratory has significantly contributed to the diagnostic and stratification of many cancer types, particularly breast cancer. The detection of both estrogen and progesterone receptors (ERs and PRs) is strongly predictive for endocrine therapy response but weak prognostic markers of clinical outcome in breast cancer [35]. Therefore, the detection of many other markers, including CXCR4, allows better evaluation of breast cancer, which can strongly influence the therapy conduct (guidance) and survival among patients.

In this particular aspect, important factors may affect IHC efficiency and reproducibility, not to mention tissue fixation (both type and duration), the choice of antibody, and the threshold for interpretation of positive immunostaining [35]. This may explain, in part, why there are controversial data regarding CXCR4 staining in different studies. Nonetheless, caution in evaluating the associative results regarding both CXCR4 and CXCL12 expressions in the tumor microenvironment is highly recommended.

In a series of elegant IHC studies with anti-human CXCR4 monoclonal antibody, CXCR4 positivity was assessed semiquantitatively using staining intensity and percentage. Li et al. [36] demonstrated that the human epithelial growth factor receptor 2 (HER2, ErbB2, and neu), which is amplified or overexpressed in about 30% of breast cancers and is a known marker of poor prognosis, enhanced CXCR4 expression and this was required for HER2-induced lung metastasis.

Kato et al. [37] examined the expression of CXCR4 protein in breast tumours, which was significantly correlated with metastatic potential of primarily involved lymph

nodes to secondary nodes. Initially, the authors suggested that the majority of breast tumors have cells with different CXCR4 expression levels, and this was directly related to the metastatic potential. However, this study also revealed that breast cancer cells express CXCR4 protein diffusely but at equal levels and that heterogeneity was rarely observed by immunohistochemical staining. Conversely, some tumours showed heterogeneous CXCR4 focal staining, which was clearly different from other cases with CXCR4 diffuse staining.

Bohn et al. [38] described a qualitatively (positive or negative) staining pattern of breast cancer using a rabbit polyclonal (1:100) antibody for human CXCR4 (cytoplasm and membrane). They did not find differences between nuclear and cytoplasmic CXCR4 expressions in primary and bone metastasis of breast cancer, and nearly all tumours showed strong immunoreactivity. Possible explanations to these findings could be related to the specific molecular mechanisms responsible for nodal metastasis, the intrinsic tumor biology, and the differences related to the antibody.

It has been suggested that CXCR4 expression in primary tumor is associated with a higher risk for bone metastasis [39]. Using anti-human CXCR4 monoclonal antibody, the immunoreactivity of membrane and cytoplasm was evaluated in breast cancer and correlated to sites of metastasis, particularly in CXCL12-producing organs. Although CXCR4 expression was not associated with clinical characteristics, prognostic for overall survival and higher rate of liver, lung, and brain metastasis, CXCR4-positive tumours showed a significantly higher risk for bone metastasis, opening new perspectives for the development of novel adjuvant strategies targeting bone tissue.

According to these findings, Sun et al. [40] have reported that CXCL12-CXCR4 axis correlated tightly with breast cancer metastasis. In this work, membrane and cytoplasmic CXCR4 staining was evaluated by IHC. Further, CXCR4 expression was significantly associated with lymph node metastasis and TNM stage. In contrast, van den Berg et al. [41] have shown that CXCR4 labeling was predominantly on the cell membrane and/or in vesicles formed after endocytosis, using confocal imaging. Deparaffinized MDAMB231 tumor tissue incubated with Ac-TZ14011-FITC showed strong non-specific staining in the nuclear membrane, nucleoli, and connective tissue. However, MDAMB231<sup>CXCR4+</sup> tumor tissue slides showed somewhat different staining profile, with cytoplasmic and membrane predominant staining.

It is already known that the GPCRs, such as CXCR4, also undergo internalization upon interaction with their respective ligand. In particular, the endocytosis induced by CXCL12 has been demonstrated in tumor cells [42]. And although this study confirmed the existence of a general regulatory mechanism of intracellular expression of the endocytosis extent, kinetics and recycling differ between cell types; it is reasonable that it has implications for traffic regulation and functional consequences [43, 44]. The predominant intracellular localization of CXCR4 suggests that the dynamic equilibrium between the plasma membrane and cytoplasm can modulate CXCR4 availability at the cell surface, which

in turn regulates cell responsiveness to follow a CXCL12 concentration gradient.

In this context, Hassan et al. [45] hypothesized that tumours overexpressing CXCR4 have an enhanced ability to metastasize in patients with low CXCL12 plasma levels. The rate of breast cancer specific mortality was higher in patients with both high phosphorylated-CXCR4 expression and low plasma CXCL12 levels than either low plasma CXCL12 or high phosphorylated-CXCR4 expression alone. Using a biotin-labeled CXCR4 antagonist, TN14003, the IHC analysis of phosphorylated-CXCR4 and CXCR4 from the tissue microarray revealed cytoplasmic and nuclear expression for both biomarkers and highlighted the prognostic value of evaluating the phosphorylated-CXCR4 expression.

The expression level and cellular localization of CXCR4 in human breast tumours have also been analyzed in view of its molecular subtypes, including triple-negative breast cancers (TNBC), luminal subtypes, and HER2-positive breast cancers, via IHC. Chen et al. [46] found that TNBC expressed CXCR4 more frequently than other subtypes, and its expression was primarily detected in the cytoplasm of tumor cells using a semiquantitative scoring system. Their study also indicated that CXCR4-positive TNBC correlated with poor clinical prognosis and was consistent with the unique visceral metastasis pattern, suggesting an effective application in TNBC treatment.

Differently, Zhang et al. [47] detected the CXCR4 protein expression by IHC in other breast cancer subtypes. These authors detected higher expression levels of the receptor in basal-like subtype, while in luminal A the expression was lower. In accordance with their study, Sivrikoz et al. [48] demonstrated that, besides basal-like subtype, the HER2 enriched breast cancer subtype also expresses high CXCR4 protein compared to the other subtypes.

Furthermore, Chu et al. [49] found that basal-like tumor patients with high CXCR4 expression had significantly higher recurrence incidence and related death than those with low CXCR4 protein expression. Hence, higher CXCR4 expression in cancer specimens might predict a worse outcome in patients with basal-like breast cancer subtype.

One of the key points on describing the role of the CXCR4/CXCL12 axis in breast cancer pathogenesis is concerned with the IHC analysis of only CXCR4, once limited information on CXCL12 expression has been published. In this regard, Papatheodorou et al. [50] investigated the immunoreactivity of CXCR4 and CXCL12 in invasive breast carcinomas and suggested that the differential CXCR4 expression at intratumoral stroma and CXCL12 in adjacent normal mammary cells may predict clinical outcome in breast cancer patients. High nuclear and cytoplasmic CXCR4 expression was detected in cancer cells. Differently, mammary epithelial cells in the adjacent nonneoplastic breast tissue showed low cytoplasmic and nuclear CXCR4 expression, in the vast majority of cases. Fibroblasts of normal stroma showed lower CXCR4 expression, while intratumoral fibroblasts were highly positive. In intratumoral endothelial cells, high CXCR4 expression was detected, while normal endothelium was negative in all cases. As far as expression patterns in normal breast tissue are concerned, results from Papatheodorou

et al. [50] are in accordance with previous studies reporting overexpression of CXCR4 in breast carcinomas compared with normal breast parenchyma [37, 51–53]. Curiously, higher CXCR4 expression in intratumoral fibroblasts was positively correlated with survival rates of patients, and both CXCL12 and CXCR4 expressions were correlated in cancer cells, intratumoral fibroblasts, and endothelial cells.

Interestingly, Aravindan et al. [54] evaluated CXCR4 and CXCL12 immunoreactivity beside cytoplasm and observed, to a lesser extent, nuclei expression in tumour epithelial cells. CXCR4 showed cytoplasmic immunoreactivity in 38.2% of tumor samples and 9.1% in normal samples using primary antibodies specific for CXCR4.

Given the diversity of findings, the difficulty in evaluating published results from different samples to the complexity of breast cancer subtypes, and the limitations of IHC analysis patterns, it is difficult to draw definitive conclusions about the role of CXCR4 in breast malignancy. However, an apparent theme is the contrast between cellular CXCR4 localization through immunohistochemical staining in tumor cell lines and breast tissue and the correlation with a more aggressive clinical outcome. It is reasonable that the role of CXCR4 in breast tumors might involve more than positive and higher expression levels in tumoral cells.

Those differences regarding positivity and localization of CXCR4 expression in breast cancer and its subtypes may represent aberrant proportion of variants, somatic mutations, or different transcriptional or cotranslational modifications [55]. Because CXCR4 represents a physiologic target for CXCL12 interaction in normal cellular signaling, it is conceivable that certain cytoplasmic staining may indicate CXCR4 internalization in response to ligand binding [41, 56]. In these cases, increased cytoplasmic CXCR4 immunostaining might abrogate membrane expression, thus limiting tumor cell evasion, and, possibly, would be associated with nonmetastatic profile.

In fact, CXCL12 expression was associated with disease-free and overall survival in a highly significant manner in two independent microarray gene expression datasets as well as in 100 breast cancer cases, analysed by IHC [57]. Compelling evidences indicate the potential protumoral, but not necessarily prometastatic, activity for CXCL12, such as proliferation mediation, apoptosis inhibition, and angiogenesis induction [27, 58].

Another important point to consider is the functional status of immune subsets within the tumor microenvironment. Heterogenous cellular CXCR4 localization must suggest divergent biological singularities in each patient, once antitumor immune response could be correlated to degrees of host immunocompetence. There is little experimental data demonstrating CXCR4 expression status in different breast tumor microenvironments, considering the mediators of inflammation related to cancer. These can induce the CXCR4 increasing expression and, depending on the circumstances, other mediators can attenuate this expression [55].

Based on these studies we conclude that there is a complex interaction between CXCR4 expressed on tumor cells with CXCL12 present in the surrounding stroma. Importantly, it is noteworthy that there is also an immunological context in

tumoral microenvironment, where often exists an infiltration of intratumoral or peritumoral immune cells also expressing CXCR4. Besides, simply quantifying the CXCR4 expression by IHC can lead to assumptions that do not evaluate the CXCR4/CXCL12 axis. Furthermore, assessment of gene expression or intracellular cytoplasmic CXCR4 detection by flow cytometry could complement the analysis by IHC and indicate how CXCR4 and CXCL12 are behaving at the molecular level, deciphering the interface of this axis in the breast cancer pathobiology.

### Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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## 4.2.ARTIGO 2

Hindawi Publishing Corporation  
Analytical Cellular Pathology  
Volume 2015, Article ID 289510, 8 pages  
<http://dx.doi.org/10.1155/2015/289510>

*Research Article*

## Genetic Polymorphism and Expression of CXCR4 in Breast Cancer

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Received 30 April 2015; Accepted 31 August 2015

Academic Editor: Monica Cantile

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*CXCR4* genetic polymorphisms, as well as their expression level, have been associated with cancer development and prognosis. The present study aimed to investigate the influence of *CXCR4* rs2228014 polymorphism on its mRNA and protein expression in breast cancer samples. It was observed that patients presented higher *CXCR4* mRNA relative expression (5.7-fold) than normal mammary gland, but this expression was not correlated with patients clinicopathological features (nuclear grade, nodal status, ER status, PR status, p53 staining, Ki67 index, and HER-2 status). Moreover, *CXCR4* mRNA relative expression also did not differ regarding the presence or absence of T allele ( $p = 0.301$ ). In the immunohistochemical assay, no difference was observed for *CXCR4* cytoplasmic protein staining in relation to different genotypes ( $p = 0.757$ ); however, high cytoplasmic *CXCR4* staining was verified in invasive breast carcinoma ( $p < 0.01$ ). All in all, the results from present study indicated that rs2228014 genetic variant does not alter *CXCR4* mRNA or protein expression. However, this receptor was more expressed in tumor compared to normal tissue, in both RNA and protein levels, suggesting its promising applicability in the general context of mammary carcinogenesis.

### 1. Introduction

Chemokines, identified on the basis of their ability to induce chemotaxis, have a fundamental role not only in inflammation and immune surveillance but also in cancer progression [1]. Chemokines, secreted by the tumor cells from primary tumors or metastatic sites or by the stromal cells recruited and/or locally activated, can behave as growth factors [2],

increase metastasis formation and angiogenesis [3], or induce the formation of an immunosuppressive microenvironment.

Chemokine receptor 4 (CXCR4) is a transmembrane receptor that belongs to the CXC chemokine receptor family and was initially reported to mediate leukocytes homing into SDF1/CXCL12 producing tissues [4]. In addition, this receptor was reported to be expressed by cancer cells [5, 6]. Many retrospective studies have documented that the expression

of various chemokine receptors, particularly CXCR4, was associated with a poor prognosis in patients with melanoma [7] and breast cancer [8].

The *CXCR4* gene is located on chromosome 2q2, in which a single nucleotide polymorphism (SNP), rs2228014 (C/T), was found at codon 138 [9, 10]. Teng et al. [11] showed that this polymorphism was associated with stages III and IV and also lymph nodes metastasis of oral cancer. Otherwise, Cacina et al. [12] have not found any significant association between *CXCR4* polymorphism and endometrial carcinoma susceptibility.

Jin et al. [13] showed that the interaction between CXCL12 secreted by endothelial cells and *CXCR4*-expressing tumor cells is sufficient to stimulate transendothelial migration. These results suggested that CXCL12/*CXCR4* axis is important in angiogenesis and tumor cell dissemination. Because both proteins were readily identifiable in a significant fraction of human breast cancer samples by immunohistochemistry, *CXCR4* may constitute a molecular target for therapy.

*CXCR4* may be overexpressed in breast cancer [14], and the *CXCR4*/*CXCL12* axis is suggested to be involved in migration and consequently in the invasion and metastasis of breast cancer cells [15]. Kang et al. [16] showed that in human breast cancer tissues the level of *CXCR4* expression is significantly correlated with lymph node metastasis and suggested that this receptor may be a useful prognostic indicator and a potential therapeutic target in breast cancer therapy.

In this context, the aim of this study was to investigate the influence of *CXCR4* rs2228014 genetic polymorphism on its gene and protein expression in breast tumor samples.

## 2. Material and Methods

Following approval from the Human Ethics Committee of State University of Londrina (CEP/UEL 189/2013-CAAE 17123113400005231), tissue samples were collected from breast cancer patients. A term of free and informed consent was signed by all sample donors and medical doctors involved prior to biologic material collection. Clinical staging was determined according to the Union of International Control of Cancer classification criteria [17]. Samples of invasive breast carcinoma tissue were obtained from 74 female patients free of adjuvant or neoadjuvant chemotherapy, who had undergone surgery at the Cancer Hospital of Londrina, Paraná, Brazil. The tumor-node-metastasis (TNM) system was used to classify the disease status 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).

**2.1. DNA Extraction from Breast Tissues.** Genomic DNA was obtained from tissue samples of invasive breast carcinoma by salting-out method [18] and was quantified by NanoDrop 2000c Spectrophotometer (Thermo Scientific Inc., Wilmington, USA) at a wavelength of 260 nm and 280 nm.

**2.2. Polymerase Chain Reaction (PCR): *CXCR4*.** DNA (100 ng) was analyzed using specific primers for *CXCR4* in a PCR reaction (GenBank accession number NM\_003467.2). Primers sequences were as follows: Forward 5'-AACTTCCTATGCAAGGCAGT-3' and Reverse 5'-TATCTGTCATCTGCC-TCACT-3'. Samples were amplified using the buffer kit plus 1.25 units of Taq polymerase (Invitrogen, Carlsbad, California, USA). PCR conditions were 5 min denaturation at 94°C, 35 cycles of 45 sec at 94°C, 1 min at 60°C and 1 min and 15 sec at 72°C, and 10 min elongation at 72°C in a Hybaid PCR Sprint Thermal Cycler (Biosystems, Guelph, Ontario, Canada). Amplicons of 236 base pairs were analyzed by electrophoresis in 2% agarose gel and visualized using UV fluorescence after staining with Blue Green reagent (LGC Biotecnologia, Sao Paulo, Brazil). All reactions were conducted with a negative control to ensure no contamination.

**2.3. *CXCR4* Genotyping.** PCR products were subjected to restriction digestion by incubating with *BclI* (New England Biolabs, UK) for 4 h at 37°C. The enzymatic restriction products were analyzed by electrophoresis on 10% polyacrylamide gel and detected by a nonradioisotopic technique using silver staining. When the allelic variant is present, a change from cytosine (C) by thymine (T) at position 3952 of the initiation codon 138 eliminates the restriction site [19]. A product of 103 and 133 base pairs for C allele and a product of 236 base pairs for T allele were observed, characterizing thereby three possible genotypes: TT (homozygous for the mutant allele), CT (heterozygous), and CC (homozygous for the wild-type allele).

**2.4. RNA Isolation and Reverse Transcriptase Reaction.** Total cellular RNA was extracted using TRIzol LS reagent (Invitrogen) according to manufacturer's instructions and quantified using NanoDrop 2000c Spectrophotometer (Thermo Scientific Inc., Wilmington, USA). Reverse transcriptase reaction was performed using 500 ng of RNA, 20 units of cloned Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT, Invitrogen), and 4 units of Recombinant Ribonuclease Inhibitor (RNaseOUT, Invitrogen) under the following conditions: 2.5  $\mu$ M oligo dT, 50 mM Tris HCl pH 8.3, 75 mM KCl, 1.5 mM MgCl<sub>2</sub>, and 1.25 mM of dNTP, at 42°C for 60 min in a Thermal Cycler.

**2.5. Real-Time PCR (qPCR) for *CXCR4*.** Quantitative real-time PCR (qPCR) was performed using Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen) on a Step One Real-Time PCR thermal cycler (Applied Biosystems, Foster City, USA). The primers used for amplification of *CXCR4* and GAPDH are described in Table 1. The qPCR reaction was performed in 40 cycles as follows: 95°C for 30 sec, 54°C for 30 sec, and 72°C for 30 sec with detection of fluorescence at each temperature increase to confirm the specific amplification. 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 (T°C)
CXCR4	AF025375	Forward	5' TGGTGGCTGAAAAGGTGGTC 3'	80.5
		Reverse	5' AAAGATGAAGTCGGGAATAGTC 3'	
GAPDH	NM_002046	Forward	5' GAAGGTGAAGGTCGGA 3'	80.5
		Reverse	5' GGGTCATTGATGGCAAC 3'	

Relative mRNA expression levels of *CXCR4* were calculated according to the  $2^{-\Delta\Delta CT}$  method [20] and normalized by the previously characterized house-keeping gene *GAPDH*. Beside adjacent normal breast tumor RNA tissue, a commercial pool of human normal mammary gland RNA (Clontech Laboratories Inc., Mountain View, CA, USA) was also used as a nonneoplastic sample.

**2.6. Immunohistochemical Staining.** For immunohistochemical analysis, 5  $\mu$ m of tissue sections was obtained from breast tumors samples. Samples were heated at 56°C, deparaffinized in xylene, and rehydrated in a graded alcohol series. Antigen retrieval was performed with citrate buffer and a mouse antibody for human *CXCR4* (1:100 dilution) (eBioscience, San Diego, CA, USA) was used. The sections were stabilized at room temperature for 30 min and washed with PBS (phosphate buffered saline) and anti-mouse/rabbit HRP secondary antibody was used as second step (Bio SB Inc., Santa Barbara, CA, USA). The diaminobenzidine (DAB) chromogen system was used (Sigma-Aldrich, USA) and counter staining was performed with Gill's hematoxylin and slide mounted in Canada balsam. The markup for *CXCR4* was assessed in tumor and adjacent normal tissue. The reading was performed under a light microscope (Eclipse-E200, Nikon, Japan) by qualified pathologists. The protocol for analysis of this marker was established at the research laboratory.

We adopted the German semiquantitative scoring system, considering the IHC staining intensity and area extent, which has been widely accepted and used in previous studies [21, 22]. Every lesion was given a score according to the intensity of the staining: weak staining = 1, moderate staining = 2, and strong staining = 3. Controls were performed to verify the specificity of primary antibody and all analyses were independently made by at least two pathologists. However, if there was a discrepancy in individual scores, both pathologists reevaluated the immunohistochemical sections by reaching a consensus agreement before combining the individual scores.

**2.7. Statistical Analysis.** One sample *t*-test was performed to analyze relative mRNA *CXCR4* expression, using GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla, California USA). Spearman correlation and Chi square statistical tests were used to analyze mRNA expression, protein expression, and rs2228014 polymorphism in relation to breast cancer clinical outcome, using SPSS Statistics 22.0 software (SPSS Inc., Chicago, Illinois, USA). A *p* value < 0.05 was considered statistically significant.

### 3. Results

In the present study, *CXCR4* rs2228014 (C/T) genetic polymorphism and mRNA expression were assessed in 74 female breast cancer patients. The median age of the patients was 58 years (ranging from 31 to 86 years old), diagnosed at the Londrina Cancer Hospital, Parana, Brazil.

The majority of patients (90.7%, 68/74) were diagnosed with invasive ductal carcinoma, according to the clinical criteria determined by the Union of International Control of Cancer [17]. The mean of tumor size was 2.7 cm  $\pm$  1.6 cm and the median size was 2.2 cm.

For genetic polymorphism assay, a PCR-RFLP methodology was performed, using the *BclI* restriction enzyme to examine *CXCR4* rs2228014 genotypes (Figure 1(a)). The obtained frequencies demonstrate that 58 (84.1%) of patients presented CC genotype, 11 (15.9%) of patients presented CT genotype, and there were no TT homozygotes (0.0%) (Figure 1(b)). The genotype distribution in our sample did not differ from the theoretical distribution given by the Hardy-Weinberg equilibrium (HWE).

No significant difference in *CXCR4* genotype distribution was observed according to clinicopathological features analyzed such as tumor histology (*p* = 0.686), nuclear grade (*p* = 0.312), nodal status (*p* = 0.697), estrogen receptor status (*p* = 0.630), progesterone receptor status (*p* = 0.287), p53 (*p* = 0.789), Ki67 (*p* = 0.129), and HER-2 status (*p* = 0.818) (Table 2).

The expression of *CXCR4* mRNA was investigated by qPCR in breast tumor tissue and in normal mammary gland. It was observed that breast cancer patients presented a higher *CXCR4* mRNA relative expression (5.7 fold) than the mRNA from normal mammary gland (Figure 2).

*CXCR4* mRNA relative expression was also assessed according to clinicopathological features, such as nuclear grade (*p* = 0.549; rho = 0.079), nodal status (*p* = 0.220; rho = -0.161), ER status (*p* = 0.745; rho = 0.042), PR status (*p* = 0.189; rho = 0.169), p53 (*p* = 0.937; rho = 0.011), Ki67 (*p* = 0.810; rho = -0.034), and HER-2 status (*p* = 0.574; rho = 0.073); however, no statistical differences were observed.

*CXCR4* mRNA relative expression was assessed in relation to rs2228014 genotypes, and the Mann-Whitney test showed no significant differences according to the presence or absence of T variant allele (*p* = 0.301) (Figure 3).

In immunohistochemical assay, although no difference was observed for *CXCR4* cytoplasmic protein levels compared to rs2228014 genotypes (*p* = 0.757), a high cytoplasmic

TABLE 2: Clinicopathological parameters analysis according to rs2228014 CXCR4 genetic polymorphism in breast cancer patients.

		Total N (%)	CXCR4 genotype		p value
			CC N (%)	Allele T carrier N (%)	
Tumor histology <sup>a</sup>	IDC	45 (90.0)	40 (80.0)	05 (10.0)	0.686
	ILC	02 (4.0)	01 (2.0)	01 (2.0)	
	Others	03 (6.0)	03 (6.0)	00 (0.0)	
Nuclear grade	I	11 (22.0)	08 (17.4)	03 (6.5)	0.312
	II	16 (34.7)	14 (30.4)	02 (4.3)	
	III	19 (41.3)	19 (41.3)	00 (0.0)	
Nodal status	Negative	27 (60.0)	22 (48.9)	05 (11.1)	0.697
	Positive	18 (40.0)	17 (37.8)	01 (2.2)	
ER status	Negative	06 (12.5)	06 (12.5)	00 (0.0)	0.630
	Positive	42 (87.5)	36 (75.0)	06 (12.5)	
PR status	Negative	10 (20.8)	10 (20.8)	00 (0.0)	0.287
	Positive	38 (79.2)	32 (66.7)	06 (12.5)	
p53	Negative	33 (75.0)	27 (61.4)	06 (13.6)	0.789
	Positive	11 (25.0)	11 (25.0)	00 (0.0)	
Ki67	Low	17 (44.8)	12 (31.6)	05 (13.2)	0.129
	Moderate	05 (13.1)	04 (10.5)	01 (2.6)	
	High	18 (42.1)	16 (42.1)	00 (0.0)	
HER2	Negative	32 (71.1)	27 (60.0)	05 (11.1)	0.818
	Positive	13 (28.9)	12 (26.7)	01 (2.2)	

<sup>a</sup>IDC: Invasive Ductal Carcinoma; ILC: Invasive Lobular Carcinoma; others: CMI, ductal carcinoma in situ.

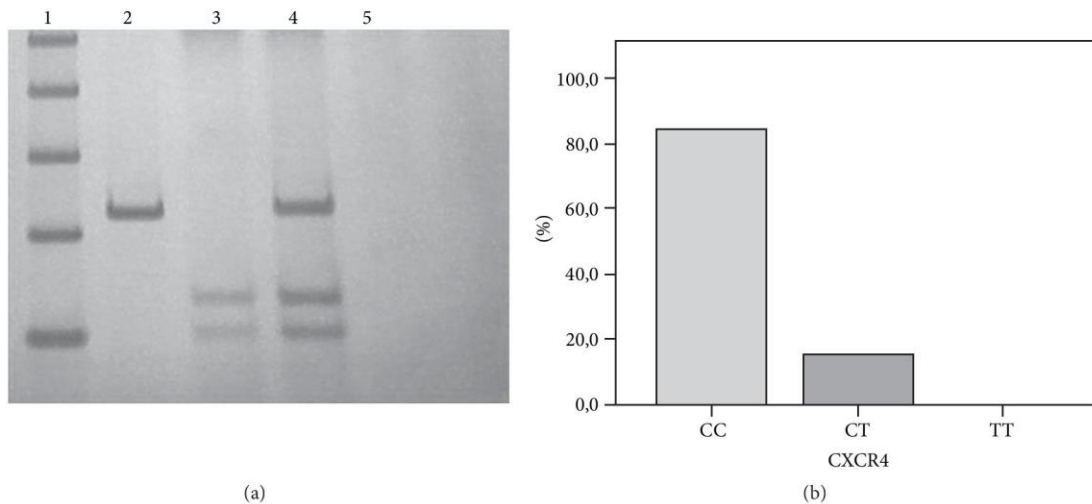


FIGURE 1: CXCR4 rs2228014 (C/T) genetic polymorphism. (a) Electrophoretic profile of rs2228014 (C/T). *BccI* restriction enzyme was used for 4 h at 37°C. Polyacrylamide gel 10% stained with silver nitrate. Lane 1, Ladder DNA fragment marker of 100 bp; Lane 2, PCR product of 236 pb; Lane 3, wild-type homozygous genotype of 133 pb and 103 pb (CC); Lane 4, heterozygous genotype of 236 pb, 133 pb, and 103 pb (CT); Lane 5, blank reaction or negative control (reaction without DNA). (b) Genotype distribution for CXCR4 rs2228014 in breast cancer patients.

CXCR4 staining was verified in invasive breast samples (Figure 4).

When CXCR4 cytoplasmic expression was verified according to breast cancer nodal status, no significant correlation was observed ( $p = 0.100$ ;  $\rho = -0.282$ ). In addition,

although CXCR4 protein expression did not change according to rs2228014 genotype distribution ( $p = 0.757$ ) (Table 3), higher protein expression in the tumor microenvironment compared with adjacent normal breast tissue ( $p = 0.01$ ) was verified.

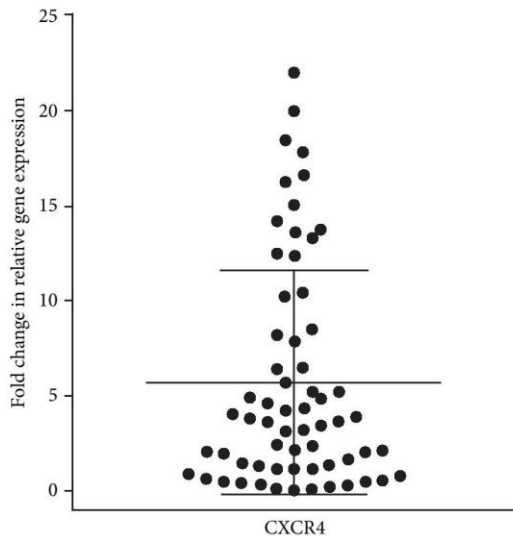


FIGURE 2: CXCR4 gene expression in tumor samples. Relative gene expression was performed by quantitative PCR using  $2^{-\Delta\Delta CT}$  method, in relation to mRNA from tumor-adjacent tissue and to normal mammary gland. Mean fold change = 5.7 ( $p < 0.0001$ ).

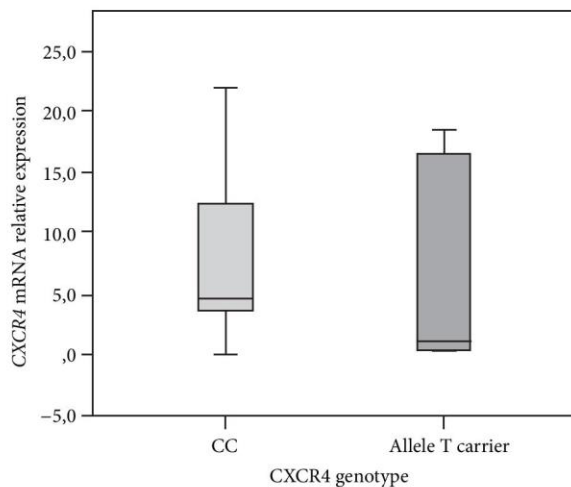


FIGURE 3: CXCR4 mRNA relative expression in accordance with rs2228014 genetic polymorphism. The Mann-Whitney test demonstrated that CXCR4 mRNA levels did not differ significantly between CC patients (mean  $7.7 \pm SE 5.65$ ) and allele T carriers (mean  $6.4 \pm SE 8.64$ ) ( $p = 0.301$ ). Error bars as 95% IC.

CXCR4 mRNA level was assessed according to its immunohistochemistry protein expression but no significant differences were observed ( $p = 0.809$ ) (Figure 5).

#### 4. Discussion

Multiple clinical, pathological, and histological features are associated with breast cancer. Fortunately, clinicopathological parameters have been validated and serve as a guide for

TABLE 3: CXCR4 protein expression according to rs2228014 genotypes.

		CXCR4 genotype	
		CC	Allele T carrier
		N (%)	N (%)
CXCR4 expression	+	09 (28.1)	02 (6.3)
	++	08 (25.0)	02 (6.3)
	+++	10 (31.2)	01 (3.1)

Pearson qui-square test;  $p = 0.757$ . +: weak, ++: moderate, and +++: strong.

systemic therapy and prognostication of breast cancer. These include tumor size, lymph node involvement, histological type, and grade and patients' age [23]. Moreover, estrogen is a growth factor that stimulates cell proliferation, and estrogen receptors (ER) mediate its effects [24]. Approximately 70% of breast cancers express the ER alpha and are hormone-dependent [25]. In accordance with this frequency, 67.1% of our samples expressed estrogen and progesterone receptors. Regarding the histological classification, 90.0% of patients presented invasive ductal carcinoma (IDC), which is in agreement with Harris and Solin [26], who observed 47–79% incidence in IDC and 2–15% invasive lobular carcinoma (ILC) in patients with breast cancer.

In addition, we investigated the effects of CXCR4 gene polymorphism on the breast cancer clinicopathological development. The analysis demonstrated that 58 (84.1%) patients presented CC genotype and 11 (15.9%) the CT genotype. No significant difference in CXCR4 genotype distribution was observed according to clinicopathological features.

Kucukgergin et al. [27] have reported that CXCR4 polymorphisms may contribute to the muscle invasive breast cancer in a Turkish population. Furthermore, Lee et al. [28] have verified that lung cancer patients carrying homozygous TT genotype of rs2228014 CXCR4 polymorphism had a tendency to develop advanced disease and poorer prognosis compared to different genotypes. Homozygous TT and heterozygous CT genotypes were also significantly associated with higher risk for renal cell carcinoma development [29].

In this work, CXCR4 genetic expression and protein detection were evaluated by real-time PCR and immunohistochemistry, respectively. It was observed that the majority of the breast cancer patients presented higher CXCR4 mRNA relative expression (5.7 fold) than mRNA from normal mammary gland and higher CXCR4 protein expression in the tumor microenvironment compared with tumor adjacent tissue. However, CXCR4 mRNA mean levels did not differ from CXCR4 immunohistochemistry status.

In this context, it has been reported that basal-like and HER2 enriched breast cancer subtypes express CXCR4 staining more often than the other subtypes. Additionally, there is also a positive relationship between lymph node involvement and CXCR4 staining of these subtypes [30]. Moreover, it is known that axillary lymph nodes positivity has been an important component for diagnosis, treatment, and subsequent research of breast cancer. Hiller et al. [31] have analyzed the literature regarding the CXCR4 expression in

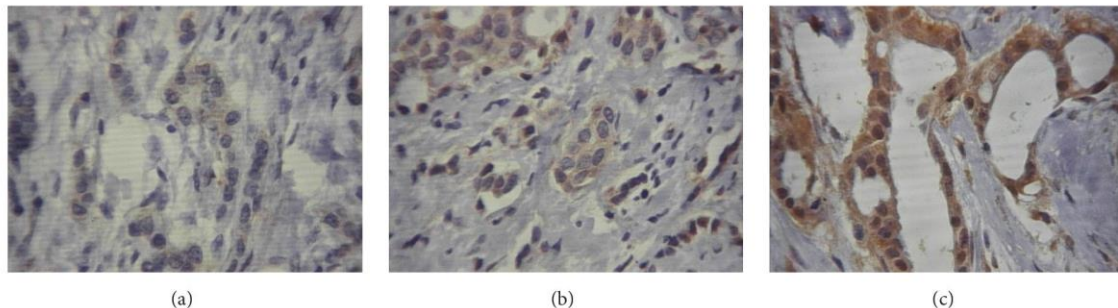


FIGURE 4: CXCR4 protein expression in breast tumor tissue samples. CXCR4 immunoreactivity was observed in the cytoplasm of tumor epithelial cells. Representative micrograph result for positive CXCR4 staining: (a) weak staining = 1, (b) moderate staining = 2, and (c) strong staining = 3. CXCR4 cytoplasmic expression in invasive breast carcinoma (400x).

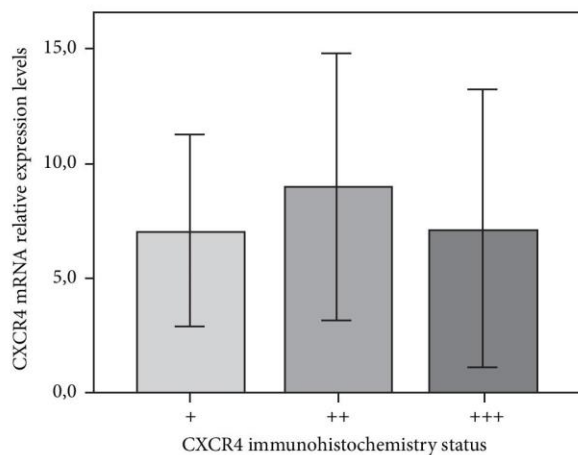


FIGURE 5: CXCR4 mRNA relative expression and immunohistochemistry status. Variance analysis tests (ANOVA) demonstrated that CXCR4 mRNA levels did not differ significantly between +/weak (mean  $7.08 \pm 6.2$ ), ++/moderate ( $8.99 \pm 7.0$ ), and +++/strong ( $7.18 \pm 7.3$ ) ( $p = 0.809$ ) immunohistochemistry statuses. Error bars as 95% IC.

breast cancer metastasis to the lymph nodes and the prognostic and/or predictive implications of lymph node metastasis in the presence of elevated CXCR4. They concluded that CXCR4 level is a predictive marker for patients with locally advanced breast cancer.

Our results showed a higher cytoplasmic CXCR4 expression staining in invasive breast carcinoma tissues through the immunohistochemical assay, although it did not differ among CXCR4 genotypes ( $p = 0.757$ ).

There is compelling evidence indicating that a subset of cancer cells, referred to as cancer stem cells, plays a critical role in tumor initiation, metastatic colonization, and resistance to therapy. Although the signals generated by the metastatic niche that regulates cancer stem cells are not fully understood, accumulating evidence suggests a key role of the CXCL12/CXCR4 axis. Cojoc et al. [32] pointed the potential

for targeting the CXCL12/CXCR4 signaling pathway in cancer management, focusing on the physiological functions of this pathway in cancer and cancer stem cells.

In this context, Sobolik et al. [33] demonstrated by intravital imaging of MCF-7 cells expressing CXCR4 that tumor cells migrate toward blood vessels and metastasize to lymph nodes. Thus, CXCR4 can drive epithelial to mesenchymal transition along with an upregulation of chemokine receptors and cytokines important in cell migration, lymphatic invasion, and tumor metastasis.

Although this study did not determine distant metastasis and relapses after treatment, considerable knowledge regarding CXCR4 role in breast cancer metastasis to CXCL12 producing organs has emerged [33–36]. In view of this function, it is reasonable to assume that the evaluation of CXCR4 expression, either at mRNA or at protein levels, could be useful as an indicator of a higher risk for metastasis. Moreover, CXCR4 should be considered for the identification of patients who are likely to develop or to prevent distant metastasis. In this regard, assessing the CXCR4 expression as a molecular breast cancer biomarker is highly demanded, and this may be performed with a standardized scoring system.

Finally, this work showed increased CXCR4 expression in breast tumor tissues, at both mRNA and protein levels, but this increase is not influenced by rs2228014 genetic polymorphism. All in all, taken together, the results from present study suggest CXCR4 receptor as a promising marker in the general context of mammary carcinogenesis.

## Conflict of Interests

The authors declare that they have no conflict of interests.

## Acknowledgments

The authors would like to acknowledge the volunteers who made this study possible and the Cancer Hospital of Londrina, Londrina, PR, Brazil. This study was supported by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Fundação Araucária, Secretaria da Ciência, Tecnologia e Ensino Superior (SETI),

and the Londrina State University Graduate Coordination (PROPPG-UEL).

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### 4.3. ARTIGO 3

#### ***TP53* polymorphism and CXCR4 expression: relation to clinicopathological features in luminal/HER2- breast cancer samples**

##### **Abstract**

Breast cancer (BC) is a heterogeneous neoplasia comprising distinct diseases, histological characteristics and clinical outcomes. *TP53* tumor suppressor has been found to be the most commonly mutated gene in many human cancers, including BC. A polymorphic site (rs1042522) at codon 72 in *TP53* encodes either an arginine (Arg) or a proline (Pro) amino acid. CXCR4 expression has been associated with a poor prognosis and metastatic process in BC. Loss of CXCR4-related p53 function has been demonstrated in tumor cells. In this context, the present study included 33 luminal/HER2- patients and 146 neoplasia free women, and examined a *TP53* polymorphism and the CXCR4 expression to verify possible gene interaction and involvement with clinicopathological features. Case control study indicated a positive association for GC heterozygous genotype ( $p=0.032$ ) and for dominant model in relation to BC susceptibility. For clinical outcome and *TP53* allelic variant correlation, it was verified a significant result for tumor size and Ki67 index. Regarding CXCR4 expression, it was observed a significant correlation to metastasis parameter. However, it was observed an absence of correlation between any *TP53* genotype and CXCR4 expression. Therefore, neither the Arginine nor Proline residues in p53 protein seem to influence CXCR4 expression. In conclusion, our findings suggest that high CXCR4 expression may be a potential marker in predicting metastasis and that codon 72 genetic variant could be a candidate for breast neoplasia susceptibility. All in all, also suggest that *TP53* codon 72 do not influences CXCR4 expression in a luminal/HER2- BC sample.

**Keyword:** *TP53*, CXCR4, breast cancer, clinicopathological features, luminal, HER2

## Introduction

Breast cancer (BC) involves a progression through a range of processes, starting with ductal hyperproliferation, followed by subsequent development of carcinoma in situ, invasive carcinoma, and finally, to metastatic disease (Polyak 2007).

According to estrogen receptor (ER) and progesterone receptor (PR) expressions, BC has different pathological and molecular clinical features (Althuis, Fergenbaum et al. 2004). It is postulated that risk factors are closely associated with estrogen and progesterone exposure, while etiology of BC ER(-) and PR(-) is independent of hormone exposure (Manjer, Malina et al. 2001) and associated with others etiological factors.

It is known that luminal tumors A and B, except luminal B-HER2 positive (erb-b2 receptor tyrosine kinase 2), are positive for ER and/or PR expressions and negative for HER2 overexpression. They are characterized by high expression of genes represented by luminal epithelial cells as cytokeratins 7, 8, 18, and 19 (Sorlie, Wang et al. 2006). Furthermore, when evaluated by immunohistochemistry, luminal A subtype present protein Ki-67 index less than 14% of neoplastic cells immunostained. Are low grade tumors and represent about 60% of breast carcinomas, been associated with a low risk of local or regional recurrence (Voduc, Cheang et al. 2010).

Mutations in the *TP53* are considered the most common genetic alterations in human cancers (Vousden and Lu 2002). A polymorphic site (rs1042522) at codon 72 in exon 4 encodes either an arginine amino acid (Arg) or a proline (Pro) residue (Matlashewski, Tuck et al. 1987). Several studies have reported epidemiological differences in the prevalence or prognostic significance of p53 mutants with arginine or proline in cancer, but the biological significance of this genetic variant remains unclear (Aoki, da Silva do Amaral Herrera et al. 2009). All in all, polymorphism rs1042522 is an important and the most studied SNP that may be associated with cancer risk (de Moura Gallo, Azevedo et al. 2005, Olivier, Hollstein et al. 2010, Alawadi, Ghabreau et al. 2011).

Regarding a new prognostic factor in BC, chemokines have been investigated in many studies. The chemokine receptor 4 (CXCR4) that is a transmembrane receptor and belongs to the CXC chemokine receptor family was reported to be expressed by cancer cells (Muller, Homey et al. 2001, Andre, Cabioglu et al. 2006) and studies have documented that CXCR4 expression was associated with a poor prognosis in BC patients (Chu, Panu et al. 2010).

Additionally, a study in cultured fibroblasts from Moskovits, Kalinkovich et al. (2006) reported that, within human and mouse fibroblasts, p53 protein can suppress the production of

CXCL12 (C-X-C motif chemokine ligand 12), a chemokine that binds to CXCR4 receptor. On the other hand, the abrogation of *TP53* expression promotes CXCL12 secretion, increasing tumor cell migration and invasiveness, showing a closed link between this tumoral suppressor gene and CXCL12/CXCR4 axis.

Knowing the dynamic inflammatory cytokine network in the human cancer, it has been showed that the loss of p53 function is a central characteristic of high-grade ovarian cancer, and CXCR4 is commonly found on these cells (Scotton, Wilson et al. 2001, Kajiyama, Shibata et al. 2008) where it is an important component of an autocrine tumour-promoting network (Kulbe, Chakravarty et al. 2012). In this context, it has been suggested that transformed ovarian surface epithelium cells may represent precursor cells of high-grade serous ovarian cancer and that CXCR4 expression may have a role in the earliest stages of this disease (Archibald, Kulbe et al. 2012).

Our research group has studied polymorphisms related to the immune system and tumor development in different cancer types. Nevertheless, there are no data relating polymorphism in *TP53* gene simultaneously with CXCR4 expression and their possible interaction and association with BC features in Brazilian population. Inside this context, the aim of the present study was to investigate the rs1042522 of *TP53* and CXCR4 expression and verify a possible involvement with clinicopathological features in luminal/HER2- BC samples.

## **Materials and Methods**

Following from the Human Ethics Committee of State University of Londrina approval (CEP/UEL 189/2013 – CAAE 17123113400005231), tissue samples were collected from BC patients. A term of free and informed consent was signed by all sample donors and medical doctors involved prior to biologic material collection.

Tissue samples were obtained from 33 female patients free of adjuvant or neoadjuvant chemotherapy, who had undergone surgery at the Cancer Hospital of Londrina (CHL), Parana State, Brazil. All patients were luminal/HER2 negative samples.

Clinical staging was determined according to the Union of International Control of Cancer classification. The tumor-node-metastasis (TNM) system was used to classify the disease status 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) (Sobin, Gospodarowicz et al. 2009, Edge and Compton 2010).

Free-neoplasia women (n=146) were invited to compose the control group. Blood samples were collected considering no personal or family history of malignancy and any evidence of BC on physical examination and mammography, at the time of sampling.

### DNA extraction

Genomic DNA from patients was obtained from the tumor tissue using salting-out method (Kirby 1993) resuspended in 30 µl milli Q water. For control group, DNA was obtained from blood samples using a specific kit for DNA extraction (Biopur, Biometrix, Curitiba, PR, Brazil) according to the manufacturer instructions. DNA samples were resuspended in 50 µl of elution buffer and all samples quantified by NanoDrop 2000c<sup>®</sup> Spectrophotometer (Thermo Scientific, Wilmington, Delaware, EUA) at a wavelength of 260/280nm. Final preparations were stored at -20°C.

### TP53 rs1042522 polymorphism

DNA was amplified by polymerase chain reaction (PCR) with specific primers, described in previous studies (Papadakis, Soultzis et al. 2002, Lima, Serafim et al. 2006, Aoki, da Silva do Amaral Herrera et al. 2009) for Tp53 codon 72 polymorphism, following the GenBank Accession: AY838896 (Table 1). DNA samples were amplified twice, with arginine and proline primers, respectively, using a heterozygous genotype sample as an amplification control.

PCR products were analyzed by electrophoresis on acrylamide gel (10%) and detected by silver staining method. TP53 C allele (Proline) allele yielded 178 base pair (pb) products, while the G allele (Arginine) yielded a 136 pb product.

**Table 1.** TP53 polymorphism primers sequences and PCR products.

Gene	Primer Sequence	PCR product
TP53 (rs1042522)	<b>Arg (G allele)</b>	
	Forward: 5' TCC CCC TTG CCG TCC CAA	136 bp
	Reverse: 5' CTG GTG CAG GGG CCA CGC 3'	
	<b>Pro (C allele)</b>	
Forward: 5' GCC AGA GGC TGC TCC CCC 3'	178 bp	
Reverse: 5' CGT GCA AGT CAC AGA CTT 3'		

bp= base pair

### RNA isolation and reverse transcriptase reaction

Total cellular RNA was extracted using TRIzol LS reagent (Invitrogen™, Carlsbad, USA) according to manufacturer's instructions and quantified using NanoDrop 2000c® Spectrophotometer (Thermo Scientific, Wilmington, USA). Reverse transcriptase reaction was performed using 500 ng of RNA, 20 units of cloned Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT; Invitrogen™, Carlsbad, USA), 4 units of Recombinant Ribonuclease Inhibitor (RNaseOUT™; Invitrogen™, Carlsbad, USA) under the following conditions: 0,4 µM oligo dT, 40 mM TrisHCl (pH 8.3), 60 mM KCl, 6,4 mM MgCl<sub>2</sub>, 0,2 mM of dNTP, at 37°C for 60 min in a Hybaid PCR Sprint Thermal Cycler.

### CXCR4 Real-Time Quantitative PCR (q-PCR)

Quantitative real time PCR (qPCR) was performed using Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen™ Carlsbad-USA) on a Step One™ Real-Time PCR Systems (Applied Biosystems, Foster City, USA). Primers used for CXCR4 and GAPDH amplifications are described in Table 2. The qPCR reaction was performed for 40 cycles as follows: 95°C for 30 sec, 54°C for 30 sec and 72°C for 30 sec with detection of fluorescence at each temperature increase to confirm the specific amplification. The comparison regarding tumoral mRNA was performed in relation to a commercial pool of normal mammary gland mRNA (Clontech Laboratories Inc., Mountain View, CA, USA)

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 2.** Quantitative RT-PCR primers sequences.

Gene	GenBank	Primer	Sequence
<i>CXCR4</i>	AF025375	<i>Foward</i>	5' TGTTGGCTGAAAAGGTGGTC 3'
		<i>Reverse</i>	5' AAAGATGAAGTCGGGAATAGTC 3'
<i>GAPDH</i>	NM_002046	<i>Foward</i>	5' GAAGGTGAAGGTCGGA 3'
		<i>Reverse</i>	5' GGGTCATTGATGGCAAC 3'

### Statistical analysis

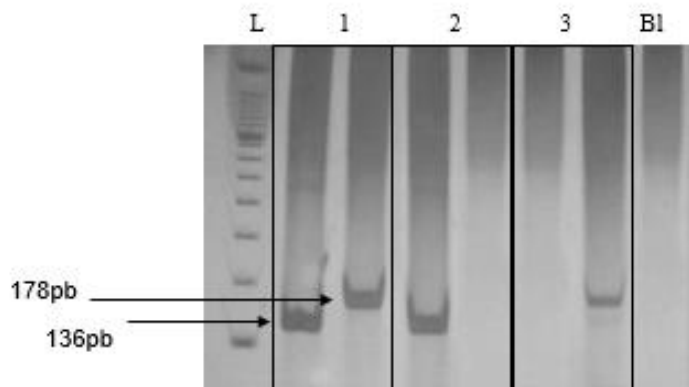
For case control study, an estimative of the relative risk at 95% confidence intervals (CI) was calculated as the Odds ratio (OR). CXCR4 gene expression in relation to genetic polymorphism was analyzed by Mann-Whitney test. Correlation analyses involving allelic

variant in relation to clinical outcome was performed by Kendall's Tau-b correlation coefficient (SPSS 20.0 Inc., Chicago, IL, USA). The  $p$  values  $< 0.05$  were considered statistically significant.

## Results

In the present study, 33 luminal/HER2- BC patients, diagnosed at the CHL, Paraná, Brazil, were investigated. The mean age of patients was  $59.5 \pm 11.92$  years and for neoplasia free women was  $54.0 \pm 13.0$  years-old ( $p > 0.05$ ). It was observed that 90.62% of patients presented ductal carcinoma, 4.69% lobular carcinoma and 4.69% other tumors. Tumor size ranged from 0.5 cm to 6.8 cm, with an average of 2.36 cm.

Electrophoretic profile for *TP53* (rs1042522) polymorphism is represented in Figure 1, which shows the PCR fragments of GC (178 bp and 136 bp), GG (136 bp) and CC (178 bp) genotypes.



**Figure 1. Electrophoretic profile for *TP53* codon 72 polymorphism.** PCR amplification of G allele (136 bp) and C allele (178 bp). Genotypes profile for heterozygote GC (lanes 1), GG homozygote (lanes 2) and CC homozygote (lanes 3) amplification are indicated. L: 100 bp ladder; Bl: Blank reaction or negative control.

Case control study indicated a positive association for GC heterozygous genotype ( $p=0.032$ ) and for C allele carriers in a dominant model ( $p=0.026$ ) of *TP53* codon 72 polymorphism in relation to BC susceptibility (Table 3).

**Table 3.** Allele frequencies, genotypes and case control study association for *TP53* polymorphism in BC luminal/HER2- patients and free neoplasia controls.

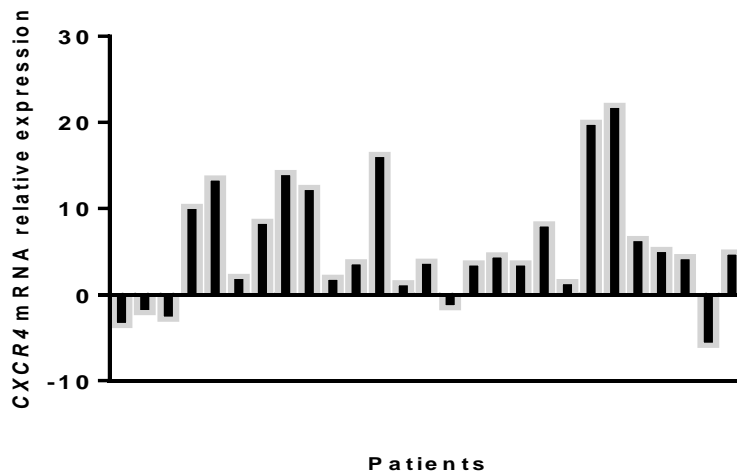
<i>TP53</i>		BC		CONTROL		OR (CI95%)	p value
		N	%	N	%		
Genotypes	GG (Arg/Arg)	14	(42.42)	93	(63.70)	1.00 (reference)	-
	GC (Arg/Pro)	16	(48.48)	41	(28.08)	2.59 (1.16-5.81)	0.032*
	CC (Pro/Pro)	3	(9.1)	12	(8.22)	1.66 (0.42-6.63)	0.44
Dominant	GG	14	(42.42)	93	(63.70)	1.00 (reference)	0.026*
	GC+CC	19	(57.58)	53	(36.3)	2,38 (1.10-5,13)	
Recessive	GG+GC	30	(90.9%)	134	(91,78)	1.00 (reference)	0.87
	CC	3	(9.1%)	12	(8,2)	1.11 (0.29-4.20)	
Allele Frequency	G (Arg)	44	66.67	227	77.74	1.75 (0.98-3.12)	0.08
	C (Pro)	22	33.33	65	22.26		

OR: Odds ratio; CI: confidence interval.

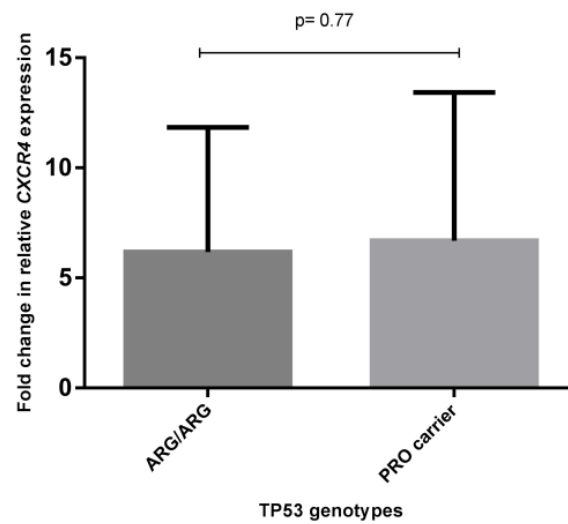
Fisher's exact test.

For most patients, CXCR4 expression was elevated in tumoral tissue in relation to normal breast tissue (Figure 2). CXCR4 expression was also increased for *TP53* genotypes in relation to normal tissue, being: 6.17 for Arg/Arg, 5.30 for Arg/Pro and 0.47 Pro/Pro genotypes. It was not observed any significant difference for CXCR4 expression between *TP53* codon 72 genotypes ( $p=0.77$ ) (Figure 3).

Regarding clinical outcome and *TP53* codon 72 genotypes, there was no significant difference for nuclear grade, p53 staining and metastasis parameters, but it was verified a significant difference in relation to tumor size and Ki67 index, a immunohistochemical prognostic factor. Regarding CXCR4 expression, a significant association was observed only between increased CXCR4 expression and metastatic involvement ( $p=0.05$ ) (Table 4).



**Figure 2. CXCR4 gene expression in tumor samples.** Relative CXCR4 gene expression was performed by quantitative PCR using  $2^{-\Delta\Delta Ct}$  method, in relation to mRNA normal mammary gland.



**Figure 3. CXCR4 mRNA relative expression in accordance with TP53 genotypes.** The Mann-Whitney test demonstrated that CXCR4 mRNA levels did not differ significantly between TP53 codon 72 genotypes ( $p=0.77$ ).

**Table 4.** Clinicopathological parameters analysis in relation to *TP53* polymorphism and CXCR4 expression in luminal/HER2- BC patients.

		<i>TP53</i> polymorphism genotypes		CXCR4 expression values	
		Arg/Arg	Pro carrier	<4	>4
		n (%)	n (%)	n (%)	n (%)
Tumor size	≤ 1.5cm	1 (3.33)	3 (10.0)	3 (11.11)	1 (4.32)
	1.6 - 3.0cm	6 (20.0)	14 (46.67)	8 (29.63)	11 (40.74)
	≥ 3.0cm	5 (16.67)	1 (3.33)	2 (7.41)	2 (7.41)
		p=0.04* τ=-0.37		p= 0.49 τ=0.14	
Metastasis	Absent	8 (26.67)	10 (33.33)	9 (34.62)	3 (11.53)
	Present	5 (16.67)	7 (23.33)	5 (19.23)	9 (34.62)
		p=0.88 τ=0.30		p=0.05* τ=0.39	
Nuclear grade	I	4 (14.29)	6 (21.43)	6 (25.0)	4 (15.38)
	II	5 (17.86)	7 (25.0)	4 (15.38)	3 (11.54)
	III	2 (7.13)	4 (14.29)	2 (13.47)	5 (19.23%)
		p=0.84 τ=-0.04		p=0.24 τ=0.25	
Ki67	Low	5 (18.52)	11 (40.74)	8 (34.78)	7 (30.43)
	Moderate	1 (3.71)	4 (14.81)	2 (8.70)	2 (8.70)
	High	6 (22.22)	0 (0.0)	2 (8.70)	2 (8.70)
		p=0.02* τ=-0.42		p=0.99 τ=0.03	
p53	Absent	11 (37.93)	14 (48.27)	11 (40.74)	13 (48.15)
	Present	2 (6.90)	2 (6.90)	2 (7.41)	1 (3.70)
		p=0.73 τ=-0.63		p=0.50 τ=-1.31	

Kendall's Tau-b, \*p&lt;0.05 values were considered statistically significant

## Discussion

Many women are still diagnosed in BC advanced stages of disease with poor evolution. Hence, an intense search for markers that may be crucial in the course of disease, especially those with prognostic and therapeutic purposes will be needed to develop personalized treatment (Banin Hirata, Oda et al. 2014).

BC is a result of collective alterations of oncogenes and tumor suppressor genes. It is well-known that p53 protein functions mainly as a tetramer transcription factor that regulates a large number of genes in response to various stresses, including ontogeny activation and DNA damage (Vogelstein, Lane et al. 2000). In the case of a mutation occurring in the *TP53* gene the protein may not only lose its normal tumor suppressor functions, but also gain new abilities that promote tumorigenesis (Brosh and Rotter 2009).

Also, mutations in this gene in BC are associated with metastasis and aggressive clinical phenotype and with poor response to systemic therapy (Berns, Foekens et al. 2000, Mehta, Christopherson et al. 2007). *TP53* codon72 polymorphism is present in a proline-rich region of the protein, and has been known to be regulating the apoptotic response (Dumont, Leu et al. 2003). It has been also suggested that the Arg72 induces apoptosis with a better efficacy than the Pro72 (Pim and Banks 2004).

(Archibald, Kulbe et al. 2012) analyzed the sequential genetic change at the *TP53* and chemokine receptor *CXCR4* locus during human ovarian surface epithelium transformation and have suggested that *CXCR4* expression and *TP53* gene alterations are intrinsically linked in ovarian cancer. In breast cancer, wild-type but not p53 mutant represses *CXCR4* expression and, on the other hand, p53 rescue drugs reduced *CXCR4* expression (Mehta, Christopherson et al. 2007).

Generally, there are conflicting data about *TP53* codon 72 and BC susceptibility. Eltahir, Adam et al. (2012) evaluated possible associations of *TP53* codon 72 polymorphism in relation to different cancers and found that BC patients most prominently showed excess of GG homozygous when compared to neoplasia-free controls. Results from Al-Qasem, Toulimat et al. (2012) indicated that the G allele was a susceptibility factor, whereas the GC (heterozygosis) form is a protection factor against BC among Saudi women. Surekha, Sailaja et al. (2011) also reported that this genetic variant might predispose to BC development as well as to worst prognosis. Damin, Frazzon et al. (2006) founded that GG genotype was significantly associated with an increased BC susceptibility, and observed no correlation in relation to prognostic predictors for disease outcome. Otherwise, Ma, Yang et al. (2011)

reported a meta- analysis, which provided strong evidence that codon 72 polymorphism was not associated with this BC development.

In the present case control study, involving a luminal/HER2- BC sample, it was observed a positive association for GC heterozygous genotype ( $p=0.032$ ) and for C allele (Pro) carriers ( $p<0,02$ ) in a dominant model, in relation to BC susceptibility (Table 3). Lopes, Guembarovski et al. (2014) demonstrated a lack of association between codon 72 polymorphism and susceptibility or prognosis of triple negative BC (TNBC). These both results from our research group may indicate that this genetic variant probably has specific BC subtype's roles. The present results may also indicate that the C allele carriers could have an effect on luminal/HER2- samples. In this context, Song, Kweon et al. (2011) have reported the association of cancer susceptibility with C allele (proline) and Arfaoui, Douik et al. (2015) with BC development. Hossain, Murshid et al. (2016) have reported that Pro/Pro homozygosity is significantly associated with BC susceptibility.

It was suggested that *TP53* mutation in cancer stem cells leads to CXCR4 up regulation (Katoh and Katoh 2010). In this context, the present study investigated codon 72 polymorphism and CXCR4 expression in BC luminal/HER2- patients and examined the possibility of CXCR4 expression been associated with *TP53* codon 72 polymorphism.

Chemokine receptor CXCR4 and its CXCL12 ligand are suggested to be involved in migration, invasion and metastasis of BC cells. CXCR4 expression has been found to be upregulated in primary tumor cells as well as metastatic lesions. The increased levels of CXCR4 expression have prognostic significance suggesting that the acquisition of the novel cancer-promoting effect is, at least in part, due to increased receptor numbers and increased receptor signals. Thus, it is essential to identify the mechanism that triggers the upregulation of CXCR4 receptor in cancer cells leading to chemotactic migration and ultimately, metastatic spread of the disease (Mukherjee and Zhao 2013). Additionally, in BC, CXCR4 expression increases progressively with malignant potential and higher expression is associated with lymph node positivity, hormone receptor negativity, Her2 overexpression and poor survival (Cabioglu, Sahin et al. 2005, Salvucci, Bouchard et al. 2006). These studies, however, did not compare CXCR4 expression with p53 status. Elevated CXCR4 expression in clinically aggressive cancers could be due to p53 mutation, both of which are associated with poor clinical outcome (Berns, Foekens et al. 2000). It was observed that patients presented higher *CXCR4* mRNA relative expression (5.7 folds) than normal mammary gland in general breast tumor samples (Okuyama Kishima, Brajao de Oliveira et al. 2015) however, the present study also showed increase of CXCR4 expression in the luminal/HER2- BC samples.

In the present study, it was not observed a significant correlation between any *TP53* genotype and CXCR4 expression. Therefore, neither the Arginine or Proline residues in the p53 protein seem to influence CXCR4 receptor expression, at least in the present sample involving a specific BC subtype.

Given the variability in clinical progression of disease, the identification of markers that could predict tumor behavior is particularly important in BC. In the present study it also was analyzed some prognostic predictors for disease outcome in relation to codon 72 polymorphism and CXCR4 expression (Table 4).

In the present study, for clinical outcome parameters and codon 72 allelic variant, it was verified a significant difference in relation to tumor size and Ki67 index. It is known that the Ki-67 antigen is a labile, non-histone nuclear protein that is tightly linked to the cell cycle and is expressed in mid-G1, S, G2, and M phases of proliferating cells but not in quiescent or resting cells of the G0 and early G1 phases. Ki-67 score is the most often measured on histological sections by IHC methodology and is defined as the percentage of stained invasive carcinoma cells (Gerdes, Lemke et al. 1984). Vielh, Chevillard et al. (1990) demonstrated a strong correlation between phase S and Ki-67 and they verified that quantitative evaluation of Ki-67 can offer a precise estimation of tumor proliferation index.

In BC, approximately 30% of patients display *TP53* gene mutation, out this frequency fluctuates from more than 80% in basal-like to less than 15% in luminal A subtypes (Sorlie, Wang et al. 2006). According to Allred, Clark et al. (1993), expression of p53 mutant protein was associated with high tumor proliferation rate, early disease recurrence, and early death in node-negative BC. The immunohistochemistry method detects this abnormal accumulation and acts as an indirect indicative of p53 mutation (Elledge, Clark et al. 1994). In the present sample of luminal/HER2- BC, there was no significant difference for p53 staining in relation to *TP53* genotypes and CXCR4 expression (Table 4). Zhang, Wang et al. (2013) reported high Ki-67 labeling index and high p53 labeling index as risk predictors of relapse for TNBC ( $p < 0.05$ ), but the findings of Kikuchi, Nishimura et al. (2013), indicated that p53 overexpression was associated with unfavorable characteristics and prognosis and appeared to be a significant prognostic factor in the patients with other molecular subtype of BC, like luminal/HER2-negative.

Since the literature has indicated CXCR4 receptor as a marker involved with the metastatic process (Mukherjee and Zhao 2013), the results of the present study, which showed a significant correlation between this receptor and metastasis parameter in a luminal/HER2- sample, corroborate the literature data and emphasize the importance of this receptor in cell

migration to tumor distant sites, as an indicator of poor prognosis.

In conclusion, our findings suggest that high CXCR4 expression may be a potential marker in predicting metastasis and *TP53* codon 72 could be a candidate marker for breast neoplasia susceptibility, but also suggest that codon 72 do not influences CXCR4 gene expression in a luminal/HER2- breast cancer sample.

### **Conflict of Interests**

The authors declare that they have no conflict of interests.

### **Acknowledgments**

The authors would like to acknowledge the volunteers who made this study possible and the Cancer Hospital of Londrina, Londrina, PR, Brazil. This study was supported by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Fundação Araucária, Secretaria da Ciência, Tecnologia e Ensino Superior (SETI), and the Londrina State University Graduate Coordination (PROPPG-UEL).

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## 5. CONCLUSÕES

### Artigo experimental 1:

- ❖ A distribuição dos genótipos do polimorfismo de *CXCR4* não apresentou diferença significativa de acordo com as características prognósticas das pacientes com câncer de mama.
- ❖ Com relação à expressão proteica de *CXCR4*, foi observada uma marcação citoplasmática mais intensa nas amostras tumorais em relação ao tecido normal adjacente;
- ❖ Não foram observadas diferenças significativas ao comparar a expressão gênica de *CXCR4* em relação à variante alélica polimórfica rs2228014, quanto à expressão proteica e em relação aos parâmetros prognósticos.

### Artigo experimental 2:

- ❖ O estudo caso controle indicou uma associação positiva para o genótipo heterozigoto e para o portador do alelo C (modelo dominante) do polimorfismo rs1042522, do gene *TP53* em relação à suscetibilidade ao câncer de mama em amostras luminal/HER2(-);
- ❖ Foi verificada uma correlação significativa para a variante genética de *TP53* em relação aos parâmetros: tamanho tumoral e índice de proliferação celular em amostras luminal/HER2- do câncer de mama;
- ❖ A expressão de *CXCR4* apresentou relação significativa com presença de metástase em amostras luminal/HER2(-);
- ❖ Os genótipos do SNP rs1042522 de *TP53* não influenciaram a expressão gênica de *CXCR4* em amostras luminal/HER2(-) do câncer de mama.

## 6. CONSIDERAÇÕES FINAIS

De um modo geral, o presente estudo mostrou um aumento da expressão de CXCR4 nos tecidos tumorais de mama, tanto em nível gênico quanto em nível proteico, o qual não foi influenciado pela presença da variante alélica avaliada no mesmo gene. Tais resultados sugerem que este receptor pode ser um marcador promissor no contexto geral da carcinogênese mamária, em especial no que se refere ao processo de metástase.

Apesar do gene *TP53* ter se mostrado associado à suscetibilidade e a parâmetros prognósticos em subtipo molecular específico de câncer de mama, o mesmo não se mostrou diretamente correlacionado ao receptor CXCR4, no presente estudo.

Assim sendo, um número maior de amostras deve ser investigado, além de se considerar o câncer de mama como doença heterogênea, constituída por vários subtipos moleculares. Havendo portanto, necessidade de avaliar os grupos de subtipos tumorais específicos para esta neoplasia, com o intuito de melhor caracterizar o papel do receptor CXCR4 nos diferentes subtipos da doença.

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## ANEXOS

## ANEXO A

Aprovação do comitê de ética em pesquisa envolvendo seres humanos da Universidade  
Estadual de Londrina



Universidade  
Estadual de Londrina



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

Parecer CEP/UEL:	189/2013
CAAE:	17123113.4.0000.5231
Data da Relatoria:	30/09/2013
Pesquisador(a):	Maria Angelica Ehara Watanabe
Unidade/Órgão:	Programa de PG em Patologia Experimental
<p>Prezado(a) Senhor(a):</p> <p>O "Comitê de Ética em Pesquisa Envolvendo Seres Humanos da Universidade Estadual de Londrina" (Registro CONEP 5231) – de acordo com as orientações da Resolução 466/12 do Conselho Nacional de Saúde/MS e Resoluções Complementares, avaliou o projeto:</p> <p>"Estudo de marcadores genéticos, epigenéticos, moleculares e imunológicos em câncer."</p>	
<p>Situação do Projeto: <b>Aprovado</b></p> <p>Informamos que deverá ser comunicada, por escrito, qualquer modificação que ocorra no desenvolvimento da pesquisa, bem como deverá apresentar ao CEP/UEL, via Plataforma Brasil, relatório final da pesquisa.</p>	
<p>Londrina, 30 de setembro de 2013.</p> <p></p> <p><b>Prof. Dra. Alexandrina Aparecida Maciel Cardelli</b> Coordenadora do Comitê de Ética em Pesquisa Envolvendo Seres Humanos Universidade Estadual de Londrina</p>	



## ANEXO B



UNIVERSIDADE ESTADUAL DE LONDRINA

### TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

#### **Informações sobre a pesquisa:**

Você está sendo convidada a participar, como voluntária, da pesquisa intitulada “**Estudo de marcadores genéticos, epigenéticos, moleculares e imunológicos em câncer**”, que tem por objetivo analisar determinados tipo de moléculas que podem influenciar na imunidade da paciente. Você será esclarecida sobre a pesquisa em qualquer aspecto que desejar. Sua participação não é obrigatória e, a qualquer momento, você poderá desistir de participar e retirar seu consentimento, sem que isso acarrete qualquer penalidade.

#### **Procedimentos do Estudo:**

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

#### **Confidencialidade da Pesquisa**

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

A amostra de sangue e tecido obtidos, serão utilizados para obtenção de DNA e RNA para a realização deste projeto. A participação no estudo não acarretará custos para você e não haverá nenhuma compensação financeira adicional. A coordenadora do projeto é a Prof<sup>a</sup>. Dr<sup>a</sup> Maria Angelica Ehara Watanabe, que pode ser encontrada no endereço: Rod. Celso Garcia cid, 445, Departamento de Ciências Patológicas, Centro de Ciências Biológicas, Universidade Estadual de Londrina, CEP: 86051-970, Tel / Fax: (43) 3371-5629, como também procurar o Comitê de Ética em Pesquisa Envolvendo Seres Humanos da Universidade Estadual de Londrina, na Avenida Robert Kock, nº 60, ou no telefone 3371 – 2490.

Pesquisador Responsável \_\_\_\_\_

RG: \_\_\_\_\_

#### **Consentimento livre esclarecido e informado:**

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

Londrina, \_\_\_\_ de \_\_\_\_\_, 20 \_\_\_\_.

Assinatura do doador (ou responsável): \_\_\_\_\_

## ANEXO C



UNIVERSIDADE ESTADUAL DE LONDRINA

### TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

#### **Informações sobre a pesquisa:**

Você está sendo convidada a participar, como voluntária, da pesquisa intitulada “**Estudo de marcadores genéticos, epigenéticos, moleculares e imunológicos em câncer**”, que tem por objetivo analisar determinados tipo de moléculas que podem influenciar na imunidade da paciente. Você será esclarecida sobre a pesquisa em qualquer aspecto que desejar. Sua participação não é obrigatória e, a qualquer momento, você poderá desistir de participar e retirar seu consentimento, sem que isso acarrete qualquer penalidade.

#### **Procedimentos do Estudo:**

Os procedimentos da pesquisa envolvem a obtenção de 5mL de sangue periférico para análise das células e moléculas do sistema imunológico.

#### **Confidencialidade da Pesquisa**

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

A amostra de sangue e tecido obtidos, serão utilizados para obtenção de DNA e RNA para a realização deste projeto. A participação no estudo não acarretará custos para você e não haverá nenhuma compensação financeira adicional. A coordenadora do projeto é a Prof<sup>a</sup>. Dr<sup>a</sup> Maria Angelica Ehara Watanabe, que pode ser encontrada no endereço: Rod. Celso Garcia cid, 445, Departamento de Ciências Patológicas, Centro de Ciências Biológicas, Universidade Estadual de Londrina, CEP: 86051-970, Tel / Fax: (43) 3371-5629, como também procurar o Comitê de Ética em Pesquisa Envolvendo Seres Humanos da Universidade Estadual de Londrina, na Avenida Robert Kock, nº 60, ou no telefone 3371 – 2490.

Pesquisador Responsável \_\_\_\_\_

RG: \_\_\_\_\_

#### **Consentimento livre esclarecido e informado:**

Eu, \_\_\_\_\_, RG \_\_\_\_\_, declaro que estou de acordo com as informações contidas neste documento, fui devidamente esclarecida pelo(s) pesquisador(es) dos objetivos e

procedimentos da pesquisa de maneira clara e detalhada, e esclareci minhas dúvidas. Concordo em participar voluntariamente como doadora saudável desse estudo sendo que poderei retirar meu consentimento a qualquer momento, antes ou durante a execução deste projeto.

Londrina, \_\_\_\_ de \_\_\_\_\_, 20 \_\_\_\_.

Assinatura do doador (ou responsável): \_\_\_\_\_