BRUNA KARINA BANIN HIRATA

ESTUDO DE ASSOCIAÇÃO DOS POLIMORFISMOS GENÉTICOS CCR2-V64I E CCR5-Δ32 COM SUSCETIBILIDADE E PARÂMETROS CLINICOPATOLÓGICOS DO CÂNCER DE MAMA

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

Orientador: Profª. Drª Maria Angelica Ehara Watanabe.
Co-orientador: Drª Roberta Losi Guembarovski.

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Londrina, 19 de fevereiro de 2014.

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BANIN-HIRATA, Bruna Karina. **Estudo de associação dos polimorfismos genéticos CCR2-V64I e CCR5-Δ32 com suscetibilidade e parâmetros clinicopatológicos do câncer de mama.** 2014. 69 f. Dissertação (Mestrado em Patologia Experimental) – Universidade Estadual de Londrina – Londrina, PR.

RESUMO

Entre os diversos tipos de cânceres, o de mama representa um grave problema de saúde pública correspondendo ao segundo tipo mais freqüente no mundo e o primeiro na população feminina. As células tumorais podem expressar receptores de quimiocinas, tais como o CCR2 e o CCR5, que estão implicados na progressão tumoral, podendo modular não apenas o recrutamento leucocitário, mas também a angiogênese, invasão e proliferação de células tumorais. Polimorfismos em muitos destes receptores são considerados fatores de risco para o desenvolvimento de diferentes tipos de câncer. A proposta deste estudo foi investigar o impacto dos polimorfismos CCR2-V64I (rs1799864) e CCR5-Δ32 (rs333) sobre a suscetibilidade e características clinicopatológicas do câncer de mama. A genotipagem foi realizada por PCR convencional e por PCR-RFLP em 118 pacientes com diagnóstico confirmado histologicamente e 180 controles livres de neoplasia de mama. O estudo de associação caso-controle foi analisado pelo cálculo da Odds Ratio (OR) com intervalo de confiança a 95% (IC=95%). E as análises de correlação entre os dados de genotipagem e os parâmetros histopatológicos (tamanho tumoral, comprometimento de linfonodos, estadiamento e grau nuclear) e subtipos tumorais do câncer de mama (triplo negativo, HER2+ e receptor hormonal positivo) foram realizadas pelo teste de Spearman rho. Nenhuma associação positiva ou negativa entre as variantes polimórficas de CCR2-V64I e CCR5-Δ32 e a suscetibilidade ao câncer de mama foi encontrada (CCR2-V64I: OR=1.32; IC95%=0.57-3.06; CCR5-Δ32: OR=1.04; IC95%=0.60-1.81). Entretanto, a análise de correlação mostrou significância entre o polimorfismo CCR2-V64I e os tumores que apresentavam a superexpressão do oncogene HER2 (p=0.026). Este estudo mostrou que os polimorfismos CCR2-V64I e CCR5-Δ32 não conferem suscetibilidade e também não estão correlacionados a um pior prognóstico no câncer de mama, em uma amostra da população brasileira da região sul. Contudo, uma vez que a freqüência dos alelos Δ32 (do gene *CCR5*) e A (do gene *CCR2*) foram baixas na população estudada (4% e 5% para o alelo Δ32 em controles e pacientes com câncer de mama, respectivamente e 12% para o alelo A em controles e pacientes), pode ser necessária a inclusão de um número maior de indivíduos com o intuito de confirmar a ausência de associação entre estes polimorfismos e a suscetibilidade ao câncer de mama. Adicionalmente, a correlação observada entre a variante alélica do gene *CCR2* com o subtipo molecular HER2+ ressalta a importância de se estudar marcadores moleculares especificamente dentro dos subgrupos do câncer de mama, dadas a heterogeneidade e variabilidade de resposta desta doença.

Palavras-chave: CCR2-V64I. CCR5-Δ32, HER2. polimorfismo genético. câncer de mama.

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ABSTRACT

Among the various types of cancer, breast cancer presents as a serious public health problem, being the second most common type in the world and first in the female population. The tumor cells can express chemokine receptors, such as CCR2 and CCR5, wich are implicated in tumor progression, can modulate not only leucocyte recruitment, but also the angiogenesis, invasion and proliferation of tumor cells. Polymorphisms of several receptors were found to be risk factors for development of different types of cancer. The purpose of this study was to investigate the impact of CCR2-V64I (rs1799864) and CCR5-Δ32 (rs333) polymorphisms on the susceptibility and clinicopathological characteristics of breast cancer. The genotyping was done by conventional PCR and PCR-RFLP methods in 118 histologically confirmed patients and 180 controls. The case-control association study was analyzed by Odds Ratio (OR) with a 95% confidence interval (IC=95%). The correlation analysis between the genotyping data and the histopathological parameters (tumor size, lymph nodes commitment, staging and nuclear grade) and breast cancer subtypes (triple negative, HER2+ and hormonal receptors positive) were realized by Spearman rho test. No association between polymorphic variants of CCR2-V64I and CCR5-Δ32 and breast cancer susceptibility was found (CCR2-V64I: OR=1.32; CI95%=0.57-3.06; CCR5-Δ32: OR=1.04; CI95%=0.60-1.81). However, the correlation analysis showed significance between the *CCR2* polymorphism and tumors with overexpression of the oncogene HER2+ (p=0.026). This study shows that CCR2-V64I and CCR5-Δ32 polymorphisms does not confers susceptibility and also are not correlated with poor prognosis in breast cancer in Southern Brazilian population sample. However, since the frequency of the Δ32 (*CCR5* gene) and A (*CCR2* gene) allele were low in the studied population (4 and 5% of Δ32 allele in controls and breast cancer patients, respectively and 12% to A allele in both groups, there may be a need the inclusion of a greater number of individuals in order to confirm the absence of association between these polymorphisms and breast cancer susceptibility in Brazilian population. Additionally, the correlation observed between the variant allele of the *CCR2* gene with the molecular subtype HER2+ highlights the importance of studying molecular markers specifically within subgroups of breast cancer, given the high heterogeneity and variability of response in this disease.

Keywords: CCR2-V64I. CCR5-Δ32. HER2. genetic polymorphism. breast cancer.

LISTA DE FIGURAS

LISTA DE ABREVIATURAS E SIGLAS

AIDS	*Acquired immunodeficiency syndrome*/ Síndrome da imunodeficiência adquirida
CCL2	*Chemokine (C-C motif) ligand 2/*Quimiocina ligante 2
CCL3	*Chemokine (C-C motif) ligand 3*/Quimiocina ligante 3
CCL4	*Chemokine (C-C motif) ligand 4*/ Quimiocina ligante 4
CCL5	*Chemokine (C-C motif) ligand 5*/ Quimiocina ligante 5
CCL7	*Chemokine (C-C motif) ligand 7*/Quimiocina ligante 7
CCL8	*Chemokine (C-C motif) ligand 8*/Quimiocina ligante 8
CCL13	*Chemokine (C-C motif) ligand 13*/Quimiocina ligante 13
CCR2	*Chemokine (C-C motif) receptor 2*/ Receptor 2 de quimiocina C-C
CCR2A	*Chemokine (C-C motif) receptor 2 isotype A*/ Receptor 2 de quimiocina C-C isótipo A
CCR2B	*Chemokine (C-C motif) receptor 2 isotype B*/ Receptor 2 de quimiocina C-C isótipo B
CCR5	*Chemokine (C-C motif) receptor 5*/ Receptor 5 de quimiocina C-C
CCR7	*Chemokine (C-C motif) receptor 7*/ Receptor 7 de quimiocina C-C
CXCR4	*Chemokine (C-X-C motif) receptor 4*/ Receptor 4 de quimiocina C-X-C
ER/ RE	*Estrogen receptor*/ Receptor de estrógeno
GPCR	*G protein-coupled receptor*/ Receptor acoplado à proteína G
HER2	*Human Epidermal growth factor receptor 2* / Receptor 2 do fator de crescimento humano epidérmico
HIV-1	*Human immunodeficiency virus type 1*/ Vírus da imunodeficiência humana tipo 1
IL-10	*Interleukin-10*/ Interleucina 10
Ki67	*Antigen Ki67* / Antígeno Ki67
MAPK	*Mitogen-activated protein kinase*/ Proteína quinase ativada por mitógeno
MDSC	*Myeloid-derived supressor cell*/ Célula supressora derivada da linhagem mielóide
MIP-1α	*Macrophage inflammatory protein-1alpha*/ Proteína inflamatória de macrófago-1 alfa
MIP-1β	*Macrophage inflammatory protein- beta*/ Proteína inflamatória de macrófago-1 beta
NK	*Natural killer cell*/ Célula assassina natural
OMS	Organização Mundial da Saúde
PGE_2	*Prostaglandin E2*/ Prostaglandina E2
PI3K	*Phosphoinositide 3-kinase*/ Fosfoinositídeo 3-quinase
PLC-β	*Phospholipase C, beta 1*/ Fosfolipase C beta 1
PR/ RP	*Progesterone receptor*/ Receptor de progesterona
RANTES	*Regulated on activation normal T cell expressed and secreted*/ Regulada sob ativação

	expressa e secretada por células T normais
Th1	*T helper type 1 lymphocyte/* Linfócito T auxiliar 1
Th17	*T helper type 17 lymphocyte*/ Linfócito T auxiliar 17
Th2	*T helper type 2 lymphocyte*/ Linfócito T auxiliar 2
TNF-α	*Tumor necrosis factor alpha*/ Fator de necrose tumoral alfa
TNM	*Tumor-Node-Metastasis Staging System*/ Sistema de estadiamento Tumor-Nódulo-Metástase
Treg	*Regulatory T cell*/ Célula T regulatória
UICC	*Union for International Cancer Control*/ União Internacional de Controle ao Câncer
VEGF	*Vascular endothelial growth factor*/ Fator de crescimento do endotélio vascular

SUMÁRIO

Introdução

Nas últimas décadas, o câncer vem adquirindo relevância crescente, convertendo-se em um evidente problema de saúde pública mundial. A Organização Mundial da Saúde (OMS) estimou que, para o ano 2030, podem-se esperar 27 milhões de casos incidentes de câncer e 17 milhões de mortes por câncer. O aumento da incidência desta doença afeta países desenvolvidos e em desenvolvimento, como resultado da crescente exposição a fatores de risco e do aumento da expectativa de vida (MINISTÉRIO DA SAÚDE [MS] e INSTITUTO NACIONAL DO CÂNCER [INCA], 2013). Segundo a Secretaria de Vigilância em Saúde as neoplasias corresponderam a terceira principal causa de mortalidade (16,4%) no Brasil em 2010, seguida apenas de causas externas (acidentes por causas diversas, suicídios e homicídios) e acidentes do aparelho circulatório (SECRETARIA DE VIGILÂNCIA EM SAÚDE, 2010).

O câncer caracteriza-se pela proliferação desregulada de células e surge a partir de alterações essenciais na fisiologia celular, as quais, coletivamente, contribuem para o crescimento dos tumores malignos. Dentre as alterações essenciais podem ser citadas: suficiência em relação aos fatores de crescimento, insensibilidade aos inibidores de crescimento, evasão à morte celular programada, potencial ilimitado de replicação, angiogênese aumentada, invasão tecidual e disseminação à distância (metástase) (HANAHAN; WEINBERG, 2000). A etiologia desta doença é genética, no entanto não é necessariamente hereditária. Os cânceres humanos são, na sua maioria, originados de mutações somáticas resultantes da interação de fatores genéticos e ambientais (PERERA, 1997).

No entanto, embora a célula tumoral represente o principal foco no desenvolvimento de uma neoplasia, é importante considerar, como enfatizado em uma revisão realizada por Kerkar e Restifo (2012), que a massa tumoral não é composta apenas de células neoplásicas, mas de um conjunto de células tumorais e elementos não neoplásicos, tais como células mesenquimais e componentes dos sistemas imune e vascular, que contribuem substancialmente para a carcinogênese, progressão tumoral e metástase das células transformadas.

Todo carcinoma humano induz uma resposta imune em seu microambiente. Geralmente, esta reação é considerada inefetiva para destruir as células tumorais. Contudo, nos últimos anos algumas evidências têm demonstrado a importância da infiltração de células do sistema imunológico, tais como linfócitos e macrófagos, no desenvolvimento de muitos tipos de câncer, através de mecanismos imunes e não-imunes (pro-angiogênico) (OSTRAND-ROSENBERG; SINHA, 2009; CRUZ-MERINO *et al*., 2013). Dentro deste contexto, as

quimiocinas (superfamília de citocinas pró-inflamatórias) e seus respectivos receptores estão implicados na progressão tumoral, modulando não apenas o recrutamento leucocitário e consequentemente na resposta imunológica tumor-específica, mas também na angiogênese, invasão e proliferação de células tumorais (BAGGIOLINI; DEWALD; MOSER, 1997; ROSSI, D.; ZLOTNIK, A. , 2000; MULLER *et al*., 2001). A interação entre as células tumorais e seu microambiente imunológico é complexa e de difícil compreensão, sendo o seu entendimento de fundamental importância para o desenvolvimento de novos marcadores prognósticos e estratégias terapêuticas para o câncer (FRIDMAN *et al*., 2011).

Câncer de mama

O câncer de mama é um grave problema de saúde pública, considerando o número de mulheres que são diagnosticadas e que morrem anualmente por esta doença. Em 2012 foram registrados cerca de 1,7 milhões de novos casos de câncer de mama (25% de todos os cânceres) e 522.000 mortes por esta causa (FERLAY *et al*., 2013). No Brasil, para o ano de 2014, são estimados 57.120 novos casos de câncer de mama. Sem considerar os tumores de pele não melanoma, esse tipo de câncer é o mais freqüente nas mulheres das regiões Sudeste (71,18/100 mil), Sul (70,98/100 mil), Centro-Oeste (51,3/100 mil) e Nordeste (36,74/ 100mil). Na região Norte é o segundo tumor mais incidente (21,29/100 mil), seguido apenas pelo câncer do colo do útero (MS/INCA, 2013).

Apesar de ser considerado um câncer de prognóstico relativamente bom se diagnosticado e tratado oportunamente, as taxas de mortalidade por câncer da mama continuam elevadas no Brasil, muito provavelmente porque a doença ainda é diagnosticada em estádios avançados. A sobrevida média na população de países desenvolvidos tem apresentado um discreto aumento, cerca de 85%. Entretanto, nos países em desenvolvimento, a sobrevida fica em torno de 60% (MS/INCA, 2011).

O curso clínico do câncer de mama e a sobrevida variam para cada paciente e dependem de uma série complexa de fatores. Os fatores de risco para esta doença incluem a idade, paridade, idade da primeira gestação, amamentação, idade da menarca e menopausa, tratamento com estrógeno, ambiente, estresse, condição imunológica e nutrição. O histórico familial é outro importante fator de risco, enfatizando o aspecto genético nesta doença (CLARK, 1996; HONDERMARCK *et al.*, 2001).

A doença metastática, caracterizada pela propagação das células tumorais através do corpo, é responsável pela maioria das mortes destes pacientes (REDIG; MCALLISTER,

2013). O câncer de mama é caracterizado por um padrão metastático distinto envolvendo linfonodos regionais, medula óssea, pulmão e fígado. A migração das células tumorais e a metástase compartilham muitas semelhanças com o tráfego de leucócitos, o qual é regulado por quimiocinas e seus receptores (MULLER *et al*., 2001). Segundo trabalho desenvolvido por Muller *et al*. (2001) os receptores de quimiocinas CXCR4 e CCR7 são altamente expressos em células tumorais de mama, mediando a polimerização de filamentos de actina e a formação de pseudópodes, subsequentemente gerando uma resposta quimiotática e invasiva, demonstrando que as quimiocinas e seus receptores desempenham um papel crítico na determinação do sítio metastático das células tumorais.

Por décadas a medicina utiliza variáveis clinicopatológicas para auxiliar no prognóstico e na escolha do tratamento dos pacientes oncológicos (BAIRD; CALDAS, 2013). O sistema de estadiamento mais utilizado para classificação dos tumores malignos é o Sistema Tumor-Nódulo-Metástase (TNM) preconizado pela União Internacional de Controle ao Câncer (UICC), o qual se baseia na extensão anatômica da doença, considerando as características do tumor primário, dos linfonodos das cadeias de drenagem linfática do órgão em que o tumor se localiza, e na presença ou ausência de metástases. A avaliação desses parâmetros permite a determinação do estadiamento que varia dos estágios 0 ao IV (SOBIN; GOSPODAROWICZ; WITTEKIND, 2009).

Atualmente, além do Sistema TNM o câncer de mama pode ser classificado de acordo com os seguintes parâmetros: tipo histológico, grau do tumor, expressão dos receptores hormonais (receptor de estrógeno [RE] e progesterona [RP]), superexpressão ou amplificação do receptor 2 do fator de crescimento humano epidérmico (HER2) e índice de proliferação celular Ki67 (SALLES *et al.*, 2009; HAMMOND *et al*., 2010; LLOYD *et al.*, 2010; VALLEJOS *et al.*, 2010). De acordo com os diferentes fenótipos obtidos, são definidos cinco subtipos do câncer de mama: luminal A (RE+, RP+, HER2-, Ki-67 baixo), luminal B (HER2 positivo [RE+ e/ou RP+, HER2+, ausência de Ki-67] e HER2 negativo [RE+ e/ou RP+, HER2- e Ki-67 alto), triplo negativo (RE-, RP-, HER2-), HER2 superexpresso (RE-, RP-, HER2+) e Basal-símile (RE-, RP-, HER-, expressão de uma ou mais citoqueratinas) (BADVE *et al*., 2011; GOLDHIRSCH *et al*., 2011).

Como descrito anteriormente, o tumor não é composto apenas de células neoplásicas, mas de um conjunto de células, incluindo as imunológicas. No câncer de mama, o sistema imune parece desempenhar um papel duplo, tanto promovendo a tumorigênese através de vias inflamatórias quanto suprimindo a imunidade adaptativa e prevenindo a formação do tumor através da vigilância imunológica (EMENS, 2012). Algumas células impedem o crescimento

tumoral, tais como; as células dendríticas, macrófagos M1, células Th1, células T $CD8^+$ e células *natural-killer* (NK), enquanto que macrófagos M2, células supressoras derivadas da linhagem mielóide (MDSCs), neutrófilos, células Th2, Th17 e T regulatórias (Tregs) promovem o crescimento tumoral. A influência destas células depende da sua distribuição intra e peritumoral, contexto imune geral e histologia do tumor de mama (FRIDMAN *et al.*, 2011). Diferentes tipos de células imunes que infiltram o tumor possuem, portanto, significância prognóstica e preditiva distinta.

Frente à importância do sistema imune no microambiente tumoral, destacam-se as quimiocinas e seus receptores que modulam o recrutamento leucocitário além de outras respostas envolvidas na progressão tumoral, tais como angiogênese e proliferação celular (BAGGIOLINI; DEWALD; MOSER, 1997; ROSSI, D.; ZLOTNIK, A. , 2000; MULLER *et al.*, 2001).

Quimiocinas e seus receptores

As quimiocinas são membros da superfamília das citocinas quimiotáticas, inicialmente caracterizadas devido a sua associação com resposta inflamatória, por estimular a quimiotaxia de leucócitos durante a inflamação (THELEN, 2001; DOWSLAND *et al.*, 2003). Contudo, hoje se sabe que as quimiocinas desempenham diversas outras funções, tais como: homeostase, proliferação celular, hematopoiese, interações vírus/célula, angiogênese, neovascularização e metástase no câncer (BAGGIOLINI; LOETSCHER, 2000; BELPERIO et al., 2000; HWANG et al., 2005; STRIETER et al., 2005; CHEN, G. S. et al., 2006)

Cinquenta quimiocinas estão descritas e todas possuem estruturas terciárias similares que consistem em um terminal amina (que atua como domínio de sinalização), seguido de uma longa alça (que contém importantes determinantes de ligação), três folhas β e uma hélice C-terminal (MURDOCH; FINN, 2000; ROSSI, D.; ZLOTNIK, A., 2000; ALLEN; CROWN; HANDEL, 2007). A maioria das quimiocinas tem quatro cisteínas conservadas e de acordo com o número de aminoácidos existentes entre os dois primeiros resíduos de cisteína da extremidade N-terminal, as quimiocinas são classificadas em quatro subfamílias: CXC, CC, CX_3C e C, onde C representa cisteína e X ou X_3 representa um ou três aminoácidos (ROSSI, D.; ZLOTNIK, A., 2000; MELLADO *et al.*, 2001; ALLEN; CROWN; HANDEL, 2007). As quimiocinas que possuem duas cisteínas adjacentes, separadas por um aminoácido (CXC) são denominadas alfa, enquanto as que possuem duas cisteínas adjacentes (CC) são denominadas beta. As quimiocinas alfa atraem neutrófilos e são produzidas por células mononucleares

ativadas, e as beta atraem macrófagos e monócitos, sendo produzidas por células T ativadas (LEVINSON, 2010). No ano 2000, um sistema de nomenclatura foi introduzido no qual cada ligante e receptor foi identificado pela subfamília recebendo um número identificador (MURPHY *et al*., 2000; BACON *et al*., 2002). Por exemplo, CCL2 refere-se a um ligante de quimiocina da subfamília CC, número 2.

As quimiocinas exercem seus efeitos através da interação com receptores de sete domínios transmembranares acoplados a proteína G (GPCR) presentes na membrana de células alvo. Atualmente, foram descritos pelo menos 20 receptores de quimiocinas e assim como no caso dos seus ligantes, os receptores também podem ser agrupados em quatro famílias principais: CR, CCR, CXCR e CX3CR, que interagem com quimiocinas C, CC, CXC e CX3C, respectivamente (MELLADO *et al.*, 2001; ZHU *et al.*, 2012). Alguns receptores, como o CCR6, CCR9, CXCR4, CXCR5, CXCR6 e CX3CR1 são específicos, ligando-se apenas a uma só quimiocina. Porém, diferentes quimiocinas podem interagir com o mesmo receptor e um único ligante pode estabelecer ligações com diferentes receptores. Estas interações ocorrem com afinidade e atividade funcional semelhantes, o que sugere um elevado grau de redundância (BALKWILL, 2004; GUERREIRO; SANTOS-COSTA; AZEVEDO-PEREIRA, 2011).

Todos estes receptores são compostos de aproximadamente 350 aminoácidos e com peso molecular próximo de 40kDa. O domínio extracelular consiste de uma extremidade N-terminal e três alças extracelulares que atuam na ligação à quimiocina. A região intracelular é composta de três alças e a extremidade C-terminal, que intervêm na transdução de sinal (MURPHY, 1994). A ligação da quimiocina ao respectivo receptor muda a conformação do mesmo e leva à dissociação das subunidades da proteína G. Estas subunidades por sua vez ativam uma variedade de vias de sinalização que envolve componentes clássicos como PLC-β, PI3K e a cascata da MAPK. Algumas destas vias podem ativar moléculas efetoras envolvidas no rearranjo do citoesqueleto, implicando na adesão e migração celular (MELLADO *et al*., 2001; FRIEDL; WOLF, 2003; SOLDEVILA; GARCÍA-ZEPEDA, 2007).

CCR2 (Receptor 2 de quimiocina C-C)

O CCR2 é um receptor de quimiocina CC que possui afinidade por CCL2, CCL7, CCL8 e CCL13 (LUSTER, 1998). Este receptor é expresso principalmente por células do sistema imune, incluindo os monócitos/macrófagos, basófilos, mastócitos, linfócitos T,

células NK e células dendríticas (CHARO *et al*., 1994; POLENTARUTTI *et al*., 1997; SANDERS *et al*., 2000) .

De acordo com uma revisão elaborada por Vicari e Caux (2002) o recrutamento dos macrófagos em tumores humanos é induzido principalmente pelo eixo CCL2/CCR2. Contudo o recrutamento destas células pode induzir tanto citotoxicidade, através da liberação de TNF-α, quanto efeitos pró-tumorais, através da produção e liberação de moléculas angiogênicas, tal como o fator de crescimento do endotélio vascular (VEGF), proteases e outros moduladores da matriz extracelular, interleucina-10 (IL-10) e prostaglandina E2 (PGE_2).

Existem duas isoformas de CCR2, o CCR2 tipo A (CCR2A) e tipo B (CCR2B), que diferem somente em sua região C-terminal (Figura 1), sugerindo que são derivados de um único gene, via *splicing* alternativo. Embora esta diferença entre as isoformas seja pequena, ela é suficiente para causar uma drástica alteração na sua localização nas células. Enquanto o CCR2B é expresso na superfície celular, o CCR2A é detectado predominantemente no citoplasma (WONG *et al.*, 1997). Todavia, quando a isoforma CCR2A é expressa na superfície celular, interage com a quimiocina CCL2 e responde à mesma de maneira similar a isoforma CCR2B (SANDERS *et al*., 2000).

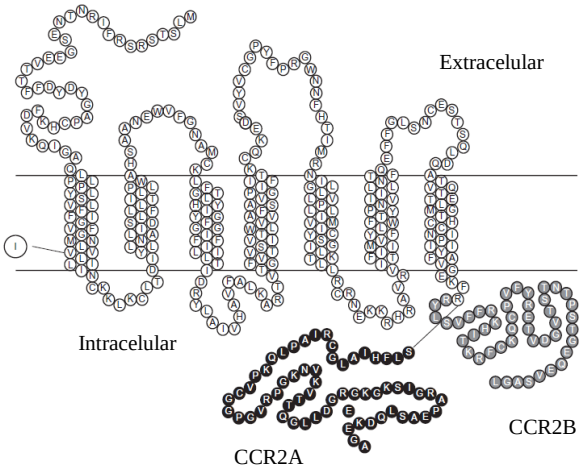

Figura 1 - Estrutura das isoformas A e B do receptor de quimiocina CCR2. Círculos em cinza representam os resíduos de aminoácidos presentes na isoforma CCR2B, enquanto que os coloridos de preto representam os resíduos de aminoácidos presentes na isoforma CCR2A. A letra I no círculo maior denota a posição onde ocorre a substituição de uma valina por uma isoleucina no polimorfismo CCR2-V64I (NAKAYAMA *et al*., 2004).

O gene *CCR2* está localizado no braço curto do cromossomo 3 (3p21) e o polimorfismo de nucleotídeo único mais estudado para este gene consiste na troca de uma guanina por uma adenina no éxon 1, na posição 190 do gene. Esta troca resulta na substituição do aminoácido valina por uma isoleucina na posição 64 da proteína CCR2 (SMITH *et al*., 1997; KOSTRIKIS *et al*., 1998), por isto este polimorfismo recebe a denotação CCR2-V64I (rs1799864).

Segundo Nakayama *et al.* (2004) o nível de expressão na superfície celular da isoforma CCR2A mutada é significativamente maior do que o do CCR2 sem a mutação. No entanto, esta substituição de valina por isoleucina não afeta os níveis de expressão da isoforma CCR2B. De acordo com este trabalho a substituição 64I aumenta a meia vida do CCR2A nas células. Em um ensaio de imunoprecipitação, estes autores demonstraram que o CCR2A co-precipitou com formas imaturas de CCR5, sugerindo que o CCR2A pode se ligar ao CCR5 no citoplasma e diminuir a sua expressão na superfície celular.

O polimorfismo CCR2-V64I está associado com aterosclerose, sarcoidose, esclerose múltipla e atraso na progressão da síndrome da imunodeficiência adquirida (AIDS) em indivíduos infectados pelo HIV-1 (LEE *et al.*, 1998; HIZAWA *et al*., 1999; MIYAGISHI *et al.*, 2003; PETRKOVA *et al.*, 2003; ORTLEPP *et al*., 2005; SHRESTHA *et al*., 2006). Além disso, Zafiropoulos *et al*. (2004) em um estudo com mulheres com câncer de mama de uma população grega, encontraram que o alelo mutado, deste polimorfismo, também pode conferir proteção contra o desenvolvimento desta doença.

CCR5 (Receptor 5 de quimiocina C-C)

O receptor 5 humano de quimiocina CC (CCR5) apresenta resposta às quimiocinas beta (MIP-1α ou CCL3, MIP-1β ou CCL4, RANTES ou CCL5) e está envolvido na quimiotaxia de determinados leucócitos para os sítios de inflamação (LIU *et al.*, 1996; MARTINSON *et al*., 1997). O CCR5 é expresso na superfície celular de linfócitos T com fenótipo de memória ou efetor, monócitos, macrófagos e células dendríticas imaturas (BLANPAIN *et al.*, 2002). Este receptor contém 352 aminoácidos com uma massa molecular de 40,6 kDa e compartilha 71% de identidade sequencial com o CCR2 (Figura 2A), onde a maioria das diferenças estão localizadas sobre os domínios extracelulares e citoplasmáticos (COMBADIERE *et al*., 1996; RAPORT *et al.,* 1996; SAMSON *et al.,* 1996). Assim como o *CCR2*, o gene *CCR5* está localizado no braço curto do cromossomo 3 (3p21) (RAPORT *et al*., 1996; SAMSON *et al.*, 1996).

O CCR5 ganhou destaque ao ser identificado como um co-receptor essencial para a entrada celular da cepa R5 do vírus da imunodeficiência humana tipo 1 (HIV-1) (ALKHATIB *et al.*, 1996; DENG *et al*., 1996; DRAGIC *et al*., 1996; FENG *et al*., 1996). O papel predominante do CCR5 para a entrada celular e replicação viral é ilustrada pela resistência ao HIV apresentada em indivíduos que perderam o CCR5 devido a uma deleção de 32 pares de base (*base pair* - bp) no gene (LIU *et al*., 1996; SAMSON *et al*., 1996; REICHE *et al*., 2008).

A deleção de 32-bp (denotado como delta CCR5 ou CCR5-Δ32) ocorre em domínios repetidos no gene *CCR5* e resulta na perda de três dos sete domínios transmembranares, duas das três alças externas e o domínio de sinalização intracelular (Figura 2B), além de uma mudança no quadro de leitura que produz uma proteína truncada, a qual não é expressa sobre a superfície celular (MCNICHOLL *et al*., 1997). Tem sido encontrada uma considerável variação na distribuição global do CCR5-Δ32. Estudos têm mostrado uma alta freqüência do alelo mutado Δ32 (aproximadamente 10%) em caucasianos; diferentemente das encontradas em outras populações, tais como: a africana, nativa americana e asiática, onde esta freqüência é inferior (GHARAGOZLOO *et al*., 2005).

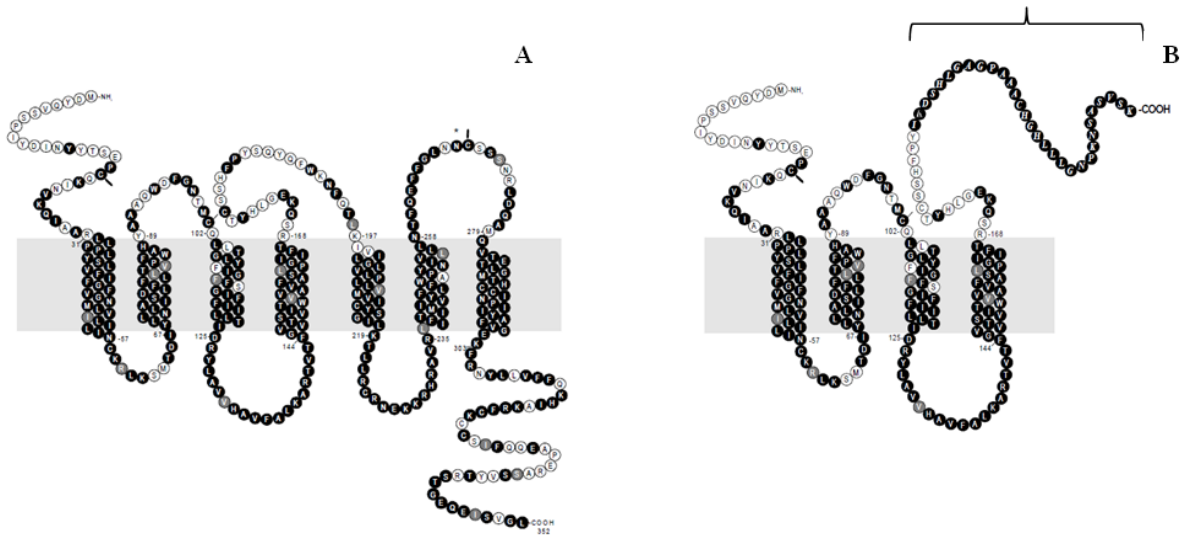

Figura 2 - Estrutura do receptor de quimiocina CCR5 selvagem e mutado. (A) A estrutura do CCR5 selvagem é composta de três alças extracelulares e intracelulares e sete domínios transmembranares. A faixa horizontal sombreada representa a membrana celular. (B) A proteína mutante perde os últimos três segmentos transmembranares, bem como as regiões envolvidas no acoplamento da proteína G. A faixa horizontal sombreada representa a membrana de organelas intracelulares. Aminoácidos indicados pela chave representam resíduos resultantes da mudança do quatro de leitura causada pela deleção (DORANZ *et al*., 1996 e SAMSON *et al*,. 1996 modificados por MCNNICHOLL *et al.*, 1997).

Além de resistência ao HIV a variante do polimorfismo CCR5-Δ32 também pode conferir proteção contra artrite reumatóide (PRAHALAD *et al.*, 2006), porém pode ser um fator predisponente para o desenvolvimento de esclerose múltipla (SHAHBAZI *et al*., 2009) e câncer de próstata (KUCUKGERGIN *et al*., 2012).

Velasco-Velazquez *et al*. (2012) relataram em seu trabalho que a sinalização CCL5/CCR5 é preferencialmente ativa nos subtipos de câncer de mama basal e HER2 positivo. Adicionalmente, estes autores demonstraram, em um segundo trabalho (VELASCO-VELAZQUEZ; PESTELL, 2013) que o CCR5 promove invasividade e metástase nas células tumorais de mama, enquanto que a inibição deste receptor protege contra estes eventos.

Objetivos

Objetivo geral

Analisar os polimorfismos genéticos dos receptores de quimiocina CCR2 e CCR5 em pacientes com câncer de mama e controles livres de neoplasia mamária, bem como sua possível correlação com parâmetros clinicopatológicos das pacientes.

Objetivos específicos

Analisar os polimorfismos genéticos CCR2-V64I (rs1799864) e CCR5-Δ32 (rs333) em pacientes com câncer de mama e em controles livres de neoplasia mamária.

Realizar um estudo de associação do tipo caso-controle para comparar a presença das variantes genéticas dos genes *CCR2* e *CCR5* entre pacientes e controles, na busca por marcadores de suscetibilidade ao desenvolvimento do câncer de mama.

Correlacionar os parâmetros clínicopatológicos das pacientes (tamanho de tumor, acometimento de linfonodos e/ou metástase à distância, grau nuclear e estadiamento clínico) com os dados genéticos na busca por marcadores ligados ao prognóstico e evolução dos tumores mamários.

Investigar uma possível correlação entre a presença das variantes genéticas com os tumores triplo-negativos, HER2 positivos e receptores hormonais positivos dentro desta amostra de câncer de mama, na busca por marcadores possivelmente relacionados aos subtipos tumorais.

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Review Article

Molecular Markers for Breast Cancer: Prediction on Tumor Behavior

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Breast cancer is one of the most common cancers with greater than 1,300,000 cases and 450,000 deaths each year worldwide. The development of breast cancer involves a progression through intermediate stages until the invasive carcinoma and finally into metastatic disease. Given the variability in clinical progression, the identification of markers that could predict the tumor behavior is particularly important in breast cancer. The determination of tumor markers is a useful tool for clinical management in cancer patients, assisting in diagnostic, staging, evaluation of therapeutic response, detection of recurrence and metastasis, and development of new treatment modalities. In this context, this review aims to discuss the main tumor markers in breast carcinogenesis. The most well-established breast molecular markers with prognostic and/or therapeutic value like hormone receptors, *HER-2* oncogene, Ki-67, and p53 proteins, and the genes for hereditary breast cancer will be presented. Furthermore, this review shows the new molecular targets in breast cancer: *CXCR4*, caveolin, miRNA, and *FOXP3*, as promising candidates for future development of effective and targeted therapies, also with lower toxicity.

1. Introduction

The global importance of cancer is unquestionable, considered the second cause of death worldwide. The incidence of different cancers had increased both in developed and in developing countries as a result of increasing exposure to risk factors and life expectancy [1]. Breast cancer is one of the most common cancers with more than 1,300,000 cases and 450,000 deaths each year worldwide [2]. In Brazil, 52,680 new cases of breast cancer are expected for 2012, with an estimated risk of 52 cases per 100,000 women [3].

Breast tumors are classified histologically according to the location of origin. The ductal tumors develop in breast ducts and represent 80% of tumors. The lobular tumors develop inside the lobes and account for 10 to 15% of cases. Other subtypes represent less than 10% of cases diagnosed per year [4]. Patients with invasive ductal carcinoma present higher lymphatic involvement and worse prognosis than less common types of breast carcinoma [5]. The staging system widely used is Tumor-Node-Metastasis (TNM) classification of malignant tumors, as recommended by the Union for International Cancer Control (UICC), which is an anatomically based system that records the primary and regional nodal extent of the tumor and the absence or presence of metastases. The evaluation of these parameters allows the determination of staging varying from stages 0 to IV [6].

The development of breast cancer involves a progression through series of intermediate processes, starting with ductal hyperproliferation, followed by subsequent evolution to carcinoma in situ, invasive carcinoma, and finally into metastatic disease [7]. Given the variability in clinical progression of disease, the identification of markers that could predict tumor behavior is particularly important in breast cancer. Also, the determination of tumor markers is a useful tool for the clinical management of cancer patients, assisting in diagnostic procedures, staging, evaluation of therapeutic

response, detection of recurrence and distant metastasis and prognosis [8], helping in the development of new treatment modalities [9]. Therefore, this review aims to discuss the main tumor markers for breast cancer development, progression and possible new therapeutic targets.

2. Molecular Markers

According to US National Institutes of Health's (NIH) Working Group and Biomarkers Consortium, a molecular marker is a characteristic that is objectively measured as an indicator of pathogenic or normal biological processes, or a pharmacological response to a therapeutic intervention [10]. Although the most of these markers is protein, recently, gene expression patterns and altered DNA identified in tumor tissue have also taken prominence as tumor markers [11].

It is known that breast cancer represents a complex and heterogeneous disease that comprises distinct pathologies, histological features, and clinical outcome. Also, it is well established that this neoplasia has well-defined molecular subgroups based on gene expression profiling closely related to the behavior of these molecular subtypes. Sotiriou and Pusztai [12] pointed out that results from studies of gene-expression profiling have altered the view of breast cancer and provided a new tool for molecular diagnosis. Actually, the status of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor type 2 (HER2) has been used as predictive markers for identifying a high-risk phenotype and for selection of the most efficient therapies [13].

The heterogeneity of breast cancer was reflected in array-CGH (comparative genomic hybridization) data of several reports, demonstrating clear or less clear associations with its subtypes [14]. After the sequencing of human genome and the technical progress in protein identification, it is reasonable considering an integrated program of genomics and proteomics to accomplish better comprehension of breast cancer features and the development of improved therapeutics [15]. Together, these results strengthened evidence of improved sensitivity and resolution methodologies, which contribute to the classification of breast cancer.

Within this context, in this review will be presented some well-stablished molecular markers of therapeutic value in the prognosis of breast cancer, and promising new markers not routinely used in clinical practice.

3. Well-Established Prognostic and/or Therapeutic Breast Cancer Markers

3.1. Hormone Receptors (HR). Approximately more than one million women are diagnosed with breast cancer each year and approximately 700.000 of these have positive (+) hormone receptors (HR) [16]. The hormone receptors are expressed proteins both in the epithelium and in breast stroma which bind to circulating hormones, mediating their cellular effects [17, 18].

The HR best studied in breast cancer are estrogen receptor (ER) and progesterone receptors (PR). Breast cancers classified by positive immunohistochemistry (IHC) expression of ER and PR have different clinical, pathological, and molecular characteristics [19]. It is postulated that risk factors are closely associated with breast tumors ER+ and PR+ and may involve mechanisms related to exposure to estrogen and progesterone, while etiology of breast cancer ER− and PR− should be independent of hormone exposure [20, 21]. The ER and PR are highly associated with patient age at diagnosis, rising continuously with age.

During the 1980s, Tamoxifen became the first anti-estrogenic therapy targeted to ER for adjuvant therapy [22]. The antagonist effects of this drug in breast tissue may result from its ability to bind to the ligand-binding domain of ER, effectively blocking the potential for estrogen stimulation. Tamoxifen binding further prevents critical ER conformational changes that are required for the association of coactivators [23]. This therapy produced clinical remission in patients with breast cancer positive for ER, differently from tumors with low or undetectable levels of these receptors [24]. Additionally, tumor cells expressing hormone receptors presented a better response to hormone therapy and patients demonstrated higher survival, both disease free as overall [25, 26], and better prognosis [27]. Although hormone therapy has revolutionized the management of breast cancer and results have improved substantially in these patients, the optimal management remains a significant challenge.

3.2. Human Epidermal Growth Factor Receptor 2 (HER-2). Several names has been given for this gene, such as c-erb-2, cerbB-2, C-erbB-2, HER-2, HER-2/neu, ERBB2, erbB2, erbB-2, neu/c-erbB-2/oncogene neu, neu protein, and neu [27]. This review will adopt the term "HER-2." The HER-2 has been extensively studied in breast cancer since Slamon et al. [28] demonstrated an association between HER-2 amplification and poor prognosis [29].

HER-2 is a transmembrane tyrosine kinase receptor belonging to a family of epidermal growth factor receptors structurally related to epidermal growth factor receptor (EGFR), encoded by *ERBB2/HER2* oncogene located on chromosome 17q21 [30]. This oncogene is amplified in 20 to 30% of breast cancers and is considered a marker of poor prognosis, once its overexpression is associated with an aggressive phenotype of tumor cells, resistance to anti-hormonal, cytotoxic therapies, and low overall survival.

In the cell signaling, the homodimerization or heterodimerization of HER family receptors activates intracellular tyrosine kinase domain which promotes the autophosphorylation of tyrosine residues of cytoplasmatic tail and thus triggers pathways that results in survival and cellular proliferation [31]. However, according to crystallographic analysis, HER-2 is ready in binding conformation even in the absence of ligand, explaining why this receptor lacks natural ligands [32].

Currently, the humanized monoclonal antibody Trastuzumab, directed against the extracellular domains of HER-2, is indicated for the treatment of HER-2 positive breast cancer

cases. The efficacy of Trastuzumab as part of an antitumor protocol has been validated in several clinical studies, where this antibody showed inhibitory effect on tumor growth and chemotherapy sensitizer [33]. Although the mechanisms by which Trastuzumab inhibits the signaling mediated by HER-2 are not fully understood, its antitumor effects are supposed to be conferred by inhibition of receptor-receptor interaction, receptor decreasing by endocytosis, blockade of extracellular domain cleavage of receptor, and activation of antibody-dependent cellular cytotoxicity (ADCC) [34, 35]. In addition to Trastuzumab, other therapeutic strategies have been developed to target HER-2 protein, such as tyrosine kinase inhibitor Lapatinib, which showed improved efficacy after failure of Trastuzumab therapy [36].

HER-2 status of breast cancer is routinely assessed by either IHC analysis of HER-2 protein or fluorescent in situ hybridization (FISH) analysis of gene copy number in primary tumor tissues. It was shown that HER-2 extracellular domain (ECD) can be shed into circulation by proteolytic cleavage from the full-length HER-2 receptor, and it is detected in serum of women with benign breast disease, primary and metastatic breast cancer [37]. The "soluble" receptor can be quantified by enzyme-linked immunoabsorbent assay (ELISA) method [38]. Tan et al. [39] established a Dot blot method to detect serum HER-2 levels, which is a valid and inexpensive assay with potential application in monitoring breast cancer progression in clinical situations.

Although HER-2 is associated with aggressive form of cancer, a specific subgroup named triple negative breast cancer (TNBC) arouses special interest, once they are orphan of directed treatment. TNBC is a subtype characterized by the lack of ER, PR, and HER-2 expression and it is associated with younger age at diagnosis [40]. There is an exhaustive search effort to find the drivers of this breast cancer subtype, because the usual antiendocrine and anti-HER2 targeted therapies are ineffective and traditional cytotoxic chemotherapy seems to be insufficient [41]. The aggressive clinical course, poor prognosis, and lack of specific therapeutic options have intensified current interest in this subtype of tumor [42]. The clinical behavior of TNBC is classically more aggressive than other types, like luminal A and B molecular subtypes, that according to Sørlie et al. [43] are considered of best and intermediate prognosis, respectively.

3.3. Ki 67 Antigen. The Ki-67 antigen, first described in 1983, is a labile, nonhistone 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 [44, 45]. Vielh et al. [46] 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. According to the St. Gallen Consensus of 2011, the proliferation index is considered low or negative, when there are 14% or less stained nuclei and it is considered positive or high, when there are more than 14% of stained nuclei [47].

Biological markers that can predict a clinic or pathologic response to primary systemic therapy of early form, during a cycle of chemotherapy, can show considerable clinical importance. Patil et al. [48] evaluated Ki-67 index and apoptotic index (AI) before, during and after neoadjuvant chemotherapy with anthracycline in indigenous woman with breast cancer, but found no significant differences.

Tawfik et al. [49] demonstrated for the first time that high expression of Ki-67 in axillary lymph nodes but not in breast tissue is significantly associated with shorter patient survival. Based on this result, patients with higher proliferative activity in lymph nodes metastases might require more aggressive therapy and closer clinical monitoring of their disease.

The prognostic and predictive value of Ki-67 was evaluated in a review developed by Luporsi et al. [50], and they concluded that this biomarker could be considered as a prognostic factor for therapeutic decision; however, standardization of techniques and scoring methods are needed for integration of this biomarker in everyday practice.

3.4. Tumor Protein p53. The p53 is involved in several critical pathways including cell cycle arrest, apoptosis, DNA repair, and cellular senescence, which are essential for normal cellular homeostasis and genome integrity maintenance. Alteration of *TP53* gene or posttranslational modification in p53 protein can alter its response to cellular stress. The molecular archaeology of *TP53* mutation spectrum generates hypotheses concerning etiology and molecular pathogenesis of human cancer [51]. In breast cancer, approximately 30% of patients display *TP53* gene mutation, but this frequency fluctuates from more than 80% in basal-like to less than 15% in luminal-A subtypes [52].

According to Allred et al. [53], expression of mutant p53 protein was associated with high tumor proliferation rate, early disease recurrence, and early death in node-negative breast cancer. Dumay et al. [54] investigated *TP53* mutations in breast tumors from the luminal, basal, and molecular apocrine molecular subgroups. They found that subgroups differ not only in *TP53* mutation frequency but also in mutation types and consequences. They detected a high prevalence of missense mutations in luminal tumors and truncating mutations in basal tumors. In apocrine molecular tumors, despite high prevalence of insertions/deletions, p53 truncation was not increased. The observations point to different mutational mechanisms, functional consequences, and/or selective pressures in different breast cancers subtypes.

Mutations in *TP53* gene result in altered molecular conformation and prolonged protein half-life leading to nuclear accumulation of altered p53 protein. The IHC method detects this abnormal accumulation and acts as an indirect indicative of mutation in *TP53* gene [55]. This nuclear accumulation is an indicator of a poor clinical outcome for breast cancer patients. However, despite its prognostic value, there is still no proper treatment that takes into account the status of this marker.

3.5. Carbohydrate 15-3 and Carcinoembryonic Antigens (CA 15-3 and CEA). Breast cancer is generally no longer curable once metastases are detected by "classical" means: clinical manifestations of the spread, imaging methods, and serum marker assays, such as those based on carcinoma antigen 15-3 (CA 15-3) or carcinoembryonic antigen (CEA) [56].

CA 15-3 in combination with CEA is also relevant tumor markers in breast cancer [57]. According to Geraghty et al. [58], the serum marker CA 15-3 has superior prognostic relevance in relation to CEA, but unlike these authors, Ebeling et al. [59] reported that prognostic value of CEA is higher than that of CA 15-3, which demonstrated that this marker has conflicting implications in breast carcinogenesis.

The CEA is a glycoprotein which has been shown to be expressed in vast majority of human colorectal, gastric, and pancreatic cancers, as well as in breast carcinomas and nonsmall cell lung carcinomas [60]. Determination of CEA in breast cancer is indicative of tumor size and nodal involvement. Therefore, CEA concentrations greater than 7.5 μg/L are associated with high probability of subclinical metastases [61]. Prognosis of patients whose CEA level was within the normal range at the time of diagnosis is significantly better than those with elevated CEA levels [62].

CA 15-3 peptides are shed or soluble forms of MUC-1, which exists as a transmembrane protein consisting of two subunits that form a stable dimer. The release has been shown to be mediated by 2 proteases, ADAM17, and MT-MMP1 [63, 64]. This is heterogeneously expressed on the apical surface of different normal epithelial cell types, but it is aberrantly overexpressed in 90% of breast cancer [65].

Sandri et al. [66] found a prognostic role for CA 15-3 within subgroups of patients with luminal B and HER-2 positive disease. According to their results, baseline CA 15-3 might be value in the identification of higher risk of relapse, where adjuvant chemotherapy must be introduced. In other words, this study showed explicitly that presence of an abnormal CA 15-3 presurgical value is associated with an increased risk of recurrence and death. Further studies using database analyses or prospective trials are required to confirm the prognostic value of presurgery CA 15-3 determination in breast cancer. If confirmed, the presence of elevated CA 15-3 should be added to the list of features that must be taken into account while making a proper treatment choice. According to Mendes et al. [67], measurement of tumor markers is a tool for detection of distant metastases, and the marker CA 15-3 seems more efficient when compared to CEA. Monitoring of breast cancer patients after surgical treatment using only this tumor marker is insufficient. However, simultaneous use of both serum markers (CA 15-3 and CEA) allows the early diagnosis of metastasis in up to 60–80% of patients with breast cancer [68].

3.6. Breast Cancer Susceptibility Genes (BRCA1 and BRCA2). Approximately 80% of the cases related to familial breast cancer are associated with one gene of hereditary susceptibility for breast and ovarian cancer, *BRCA1* and *BRCA2*. The *BRCA* genes have been classified as tumour-suppressor genes, because the loss of wild-type allele has been observed in tumors of heterozygous carriers. BRCA proteins play important roles in different cellular processes, including activation and transcriptional regulation, repair of DNA damage, beyond the control of cell cycle, cellular proliferation, and differentiation [69, 70].

Besides breast cancer, these genes are associated with elevated risk of ovary, prostate, and pancreas cancers. However, despite its association with inherited predisposition, somatic disease-causing mutations in *BRCA1* or *BRCA2* are extremely rare in sporadic breast cancer [71, 72].

The frequency and spectrum of mutations within *BRCA1* or *BRCA2* genes show considerable variation between ethnic group and geographic region, probably due to interactions between different lifestyle and genetic characteristics. Studies have discussed the role of maternal or paternal inheritance of *BRCA* mutation affecting risk of breast cancer. Shapira et al. [73] showed that lifetime risk was higher in *BRCA* mutation inherited from the father, compared to the mother. However, in accordance to Senst et al. [74], although the risk of breast cancer seems to be modestly higher in women with paternal *BRCA1* mutation, the results of the study were not significant. Thus, data are not sufficiently compelling to justify different screening recommendations for the two subgroups. Furthermore, parental mutation origin also did not affect the risk in women with *BRCA2* mutation.

Family history profiles can predict *BRCA1* or *BRCA2* mutation, mainly those characterized by first-degree relatives with ovarian cancer or breast cancer along with young age at diagnosis, bilateral occurrence and increased number of affected relatives. These predictors would be useful in genetic counseling and decision-making for a genetic test but they are still of limited value since a considerable number of *BRCA1* or *BRCA2* mutations are observed in breast cancer families without such risk factors. Definitive predictors need to be developed in future studies [75].

Genetic counseling and genetic testing to identify *BRCA1* and *BRCA2* gene mutations in high-risk patients are widely available and commonly employed in the US and Europe [76, 77]. Individuals who undergo genetic testing by sequencing their DNA for specific regions of these genes and discover that they carry a *BRCA* mutation can have the diagnosis of breast cancer anticipated and perhaps in some cases prevented. If a high-risk status of these women had been recognized, they might have had the opportunity to choose genetic counseling, testing, more effective cancer surveillance, and potentially preventive options, such as prophylactic surgery and/or chemoprevention [78]. Among these preventive options, bilateral mastectomy, although invasive, reduces approximately 90% the risk of breast cancer in women with *BRCA1/2* mutations [79].

According to Apostolou and Fostira [80], more susceptible genes have been discovered and BRCA1 and BRCA2 predisposition seems to be only a part of the story. These new findings include rare germline mutations in other high penetrant genes; the most important between them include TP53 mutations in Li-Fraumeni syndrome, STK11 (serine/threonine kinase 11) mutations in Peutz-Jeghers syndrome, and PTEN (phosphatase and tensin homolog on chromosome ten) mutations in Cowden syndrome. Furthermore,

more frequent, but less penetrant, mutations have been identified in families with breast cancer clustering, in moderate or low penetrant genes, such as CHEK2 (checkpoint kinase 2), ATM (ataxia telangiectasia mutated), PALB2 (partner and localizer of BRCA2), and BRIP1 (BRCA1-interacting protein C-terminal helicase 1).

4. Future Candidate Markers for Prognosis and Therapeutic Responses in Breast Cancer Evolution

4.1. Proliferating Cell Nuclear Antigen (PCNA). Moldovan et al. [81] have described the proliferating cell nuclear antigen (PCNA) as a nonhistone nuclear protein that forms a homotrimeric ring encircling DNA double helix and acts as a molecular platform to recruit proteins involved in DNA synthesis, such as DNA polymerase delta, cell-cycle control, DNA-damage response, and repair. PCNA exists in two distinct forms: replication-competent chromatin-bound form and chromatin unbound form, which is not involved in DNA synthesis [82]. For instance, it is not clear how these two populations of PCNA are regulated. In a number of tumors, measurement of this protein was associated with mitotic activity and tumor grade [83]. The PCNA signal transduction has an important impact on growth regulation of breast cancer cells and is associated with poor overall survival [84]. Recently, it was reported that phosphorylation of PCNA at tyrosine 211 (Y211) is a promising treatment target in prostate cancer [85]. The results obtained by Zhao et al. [84] suggested that targeting phospho-Y211 PCNA could be an effective strategy in breast cancer treatment as well in the future.

4.2. Caveolin. Studies showed that caveolae and caveolin 1 play an essential role in many molecular, cellular, and physiological processes [86]. Caveolae are special invaginated microdomains of the plasma membrane found in the majority of mammalian cells and serve as membrane organizing centers. Three members of caveolin family (CAV1, CAV2, and CAV3) have been identified and they play a pivotal role in intracellular trafficking of cellular components and in signal transduction [87].

Recently, it has been reported an strong association between CAV1 and CAV2 expression and high histological grade, and lack of hormone receptors positivity (ER and PR) in basal-like breast cancer subtype [88], providing evidence that these proteins can have oncogenic properties. Aside from breast, association between caveolin expression and poor patient outcome was noticed in other tumor tissues, including prostate [89], lung [90], and central nervous system [91].

The caveolin 1 influences cancer formation, progression, and prognosis, but this influence is not so sharp, in spite of recent results that have clarified many roles. Its role as oncoprotein or tumor suppressor may depend on interaction with molecular signaling molecules by specific regions, and this may be modified by genetic changes, mRNA, and protein expression level. Current and future research into mechanisms by which caveolin 1 function in tumorigenesis process will most likely lead to a new molecular marker in diagnosis and prognosis and even in treatment of breast cancer [86].

4.3. Receptor C-X-C Chemokine Receptor Type 4 (CXCR4). The CXCR4 is transmembrane G-coupled receptor protein, identified as a coreceptor for T-cell line tropic strains of human immunodeficiency virus [92]. Its role in breast cancer metastasis was first documented in 2001 [93].

This receptor is required for migration of breast cancer cells from the primary site to lung, bones, and lymph nodes, which represent organs that secrete high levels of chemokine CXCL12. For this reason, CXCR4 has been found to be a prognostic marker in breast cancer, among other types of cancer [94].

Schioppa et al. [95] made an important observation that the expression of CXCR4 is up-regulated in tumor cells resulting from a change in tumor microenvironment. Tumor cells cultured in hypoxic conditions, for example, showed significant overexpression of CXCR4.

The expression level of CXCR4 is significantly correlated with lymph node metastasis [96]. Patients who had CXCR4 overexpression had significantly higher incidence of cancer recurrence and cancer-related deaths than those in low CXCR4 expression group [97]. Its expression also is significantly related to tumor size, advanced TNM stage, and shorter overall- and disease-free survival. However, in luminal or HER2-positive breast cancer groups, CXCR4 was not correlated with such clinic-pathological characteristics and survival. This association is cardinal in TNBC patients who expressed high levels of CXCR4, which have poorer disease-free survival and overall survival, compared with TNBC patients expressing low levels of this marker [98]. These findings indicated that CXCR4 high expression in TNBC might indicate a more aggressive tumor phenotype.

Therefore, this receptor may be a potential therapeutic target in cancer therapies for breast cancer. So far, the best studied among the compounds that inhibit CXCR4-CXCL12 interaction is the antagonist AMD3100. This compound significantly inhibits the invasion and metastasis activity of cancer cells [99].

4.4. Chemokine (C-C Motif) Ligands 2 and 5 (CCL2 and CCL5). Many studies have addressed the involvement and roles of inflammatory chemokines CCL2 (MCP-1, membrane cofactor protein-1) and CCL5 (RANTES, regulated on activation, normal T-cell expressed and secreted) in breast malignancy. Belonging to chemokine super family, CCL2 and CCL5 are well recognized because of their activities in the immune context, where they induce leukocyte directed motility. Acting mainly in inflammatory reactions, these two chemokines stimulate migration primarily of monocytes and T-cells to damaged or infected sites [100–102].

Some studies have shown that CCL2 and CCL5 expressions are higher in breast cancer tissue than in corresponding normal tissue [103, 104]. CCL2 has been correlated with higher tumor grade and has been shown to have significant prognostic value for relapse-free survival. The CCL2

likely exerts its protumorigenic effects through recruitment of tumor-associated macrophages and angiogenesis [105]. Furthermore, CCL2/CCR2 (CCL2 receptor) chemokine signaling seems to be implicated in cell migration and its overexpression is associated with breast cancer metastasis to both lung and bone [104, 106].

The CCL5/CCR5 (CCL5 receptor) axis is active in patients affected by aggressive basal subtype of breast cancer. Murooka et al. [107] showed that CCL5 enhanced MCF-7 (breast cancer cell lines) proliferation. Furthermore, according to Velasco-Velazquez and Pestell [108], CCR5 promoted breast cancer invasiveness and metastatic potential. These results indicated that CCL5 expression is directly correlated with more advanced stage of disease, emphasizing their involvement in breast cancer progression [109]. In this context, these two chemokines could be considered as prognostic markers and therapeutic targets for breast cancer.

4.5. Growth Factors: EGF, HGF, IGF, VEGF, and TGF-β. The role of growth factors has been extensively analyzed both in cancer risk and tumor progression. Cerna et al. [110] found a negative correlation between insulin-like growth factor I (IGF1) and severity of cancer. Thus, according to these authors, this growth factor cannot be used for quick and correct orientation in clinical condition of patients in the early stages of tumor growth, unlike epidermal growth factor (EGF). Nevertheless, the IGF1 and EGF are stimuli to migration of cancer cells to distant areas, to form metastasis, and have been implicated in the development and progression of human breast carcinoma [111].

The hepatocyte growth factor (HGF) is considered a progression and aggressiveness marker of breast cancer and data obtained by Kucera et al. [112] fully corresponds to this. Based on their data, this marker could potentially be used as an additional tool for the differentiation between benign and malignant tumor. Ahmed et al. [113] also demonstrated that serum levels of HGF may help in the diagnosis of breast cancer patients and may aid in disease prognosis. However, Cerna et al. [110] related the opposite for HGF as well as IGF1 and vascular endothelial growth factor (VEGF). It was demonstrated that tumor stromal VEGF-A expression is a valuable prognostic indicator of breast cancer-specific and disease-free survival at diagnosis and can therefore be used to stratify patients with inflammatory breast cancer (IBC) into low-risk and high-risk groups for death and relapses. Furthermore, high levels of tumor stromal VEGF-A may be useful to identify IBC patients who will benefit from antiangiogenic treatment, since VEGF-A is the most potent promoter of angiogenesis and lymphangiogenesis [114].

Conflicting results have been published about the transforming growth factor-β (TGF-β). Oda et al. [115] showed that homozygous patients for CC genotype from T869C polymorphism presented a higher TGF-β expression and suggested a role of this gene as progression marker for breast carcinoma. It is known that overexpression of TGF-β by both tumor and stromal tissue can facilitate the development of metastasis, mainly in behalf of TGF-β stimuli to angiogenesis and increased tumor cell motility [116]. According to Sheen-Chen et al. [117], high TGF-β1 serum levels have been associated with advanced stages of breast cancer; thus, it may reflect the severity of invasive breast cancer. However, Figueroa et al. [118] found that TGF-β expression was correlated with favorable prognostic features. These results are seemingly discordant and possibly represent the dual role of TGF-β in cancer development, in which it displays both tumorigenic and tumor-suppressive effects. de Kruijf et al. [119] claimed that combining TGF-β biomarkers provides prognostic information for patients with stage I–III breast cancer. The authors believed that this marker could identify patients at increased risk for disease recurrence that might therefore be candidates for additional treatment.

4.6. V-Myc Myelocytomatosis Viral Oncogene Homolog (Avian) (MYC). The *MYC* proto-oncogene family (comprising c-myc, N-myc, and L-muc) ranks among the most exhaustively studied group of genes in biology. MYC is a basic helix-loop-helix zipper (bHLHZ) protein whose activity is tightly regulated by its direct binding to another bHLHZ protein MAX. *MYC* activation can lead to transcriptional activation or repression of specific genes [120]. This gene seems to have an important role in carcinogenesis and tumor replication, growth, metabolism, differentiation, and apoptosis [121].

Amplification of *MYC* has been reported in breast cancer as well as in many other cancers. However, amplification of this gene is not established as prognostic or predictive factor yet because there are many inconsistent results [122, 123]. Notwithstanding, several converging studies have suggested that *MYC* may play an important function in breast cancer.

A recent report from Horiuchi et al. [124] found that TNBC tumors exhibit elevated *MYC* expression, resulting in increased activity of the *MYC* pathway. They showed that CDK inhibition effectively induced tumor regression, indicating that aggressive breast tumors with elevated *MYC* expression are uniquely sensitive to CDK inhibitors.

MYC amplification shows strong correlation with ER status, stage of disease (initial), and existence of distant metastasis and tends to be associated with high histologic grade, positive axillary nodal status, and a high S-phase fraction. Furthermore, its amplification is not significantly associated with overall survival of patients with invasive cancer. Thus, this genetic alteration is a feature of aggressive breast cancer, but is unlikely to be a clinically useful prognostic factor [122, 125].

Thus, despite that the expression of *MYC* is significantly different between breast cancer patients and healthy controls [126], correlation between *MYC* amplification and different clinicopathological parameters are inconsistent. Regardless of lack of evidence for the prognostic significance of *MYC* amplification, it could represent a clinically useful predictive parameter in metastatic breast cancer [122].

4.7. Forkhead Box Protein 3 (FOXP3). Forkhead box protein P3 (Foxp3) plays a critical role in differentiation, development, maintenance, and function of regulatory T-cells

(Treg) [127]. However, it does not necessarily confer a Treg phenotype when expressed in CD4+ T lymphocytes. High Treg levels have been reported in peripheral blood, lymph nodes, and tumor specimens from patients with different types of cancer. The precise mechanisms by which Tregs suppress immune cell functions remain unclear, and there are reports of both direct inhibition through cell-cell contact and indirect inhibition through the secretion of anti-inflammatory mediators such as interleukin. Signals from the microenvironment have a profound influence on the maintenance and progression of cancers. Although T-cells present the most important immunological response in tumor growth in the early stages of cancer, they become suppressive CD4+ and CD8+ regulatory T-cells after chronic stimulation and interactions with tumor cells, thus promoting rather than inhibiting cancer development and progression [128].

It was recently demonstrated that FOXP3 is expressed at both mRNA and protein levels in the nucleus of epithelial cells in prostate [129], breast [130, 131], and lung [132]. It is already becoming clear that cancer cells can show dysregulated FOXP3 expression. Several studies have examined the distribution of FOXP3 in normal and malignant epithelial breast cells [131, 133]. Each study has consistently reported that FOXP3 is expressed constitutively within the nucleus of healthy epithelial cells. However, description of FOXP3 localization in cancerous epithelia is less definitive. Some reports showed a nuclear location similar to that observed in healthy cells whereas others describe either a complete absence of FOXP3, mutations or a change in subcellular distribution [129, 131, 133]. When breast cancer survival rate was correlated with location of this marker, patients with FOXP3 restricted to cytoplasm had similarly poor prognosis than patients with no detectable FOXP3 [131]. These data suggested that failure recruitment of FOXP3 to the nucleus could act as an important prognostic marker associated with a more aggressive form of breast cancer and poor survival. Similarly, almost 20% of breast cancer cells and 80% of non-malignant cells expressed nuclear FOXP3, when assessing subcellular distribution of FOXP3 in breast cancer patient samples [133].

FOXP3 is able to repress the expression of MYC [129] and in normal breast epithelium is able to bind to and repress the expression of HER-2 [133], with prognostic relevance [33].

Overbeck-Zubrzycka et al. [134] demonstrated that normal breast epithelia expressed FOXP3 constitutively within nucleus and failed to express CXCR4, whereas breast cancer samples and breast cancer metastasis expressed diminished levels of nuclear FOXP3 and also expressed significantly higher levels of membrane CXCR4. The increased expression of CXCR4 on these cells allows a potent response to CXCL12 and migration to site-specific regions of the body [135, 136].

4.8. microRNA. Over the past decades, an increasing amount of evidence has demonstrated applications of microRNAs (miRNAs) as tissue based markers for classification and prognosis of several human cancers, including breast cancer [137, 138].

miRNAs are naturally occurring, noncoding small RNA molecules of 21–24 nucleotides that binds partially or completely to 3′untranslated regions (3′-UTRs) of protein-coding genes, leading to cleavage or translational repression of targets [139, 140]. The miRNA can be stably present in whole blood, serum, and plasma [141], but the origin of circulating miRNA is still unclear. It has been proposed that tumor-associated miRNAs can be released into bloodstream when tumor cells are dying and being lysed [142] or through active secretion of miRNA loaded exosomes by tumor cells [143].

Some studies have indicated that microRNAs play a pivotal role in most critical biological events, including development, proliferation, differentiation, cell fate determination, apoptosis, signal transduction, organ development, hematopoietic lineage differentiation, host-viral interactions, and tumorigenesis [144, 145].

Wu et al. [146] detected more than 800 miRNAs in the circulation of breast cancer patients. Two of them, miR-375 and miR-122, exhibited strong correlation with clinical outcomes, including neoadjuvant chemotherapy response and metastatic relapse. These results may allow optimized chemotherapy treatments and preventive antimetastasis intervention in future clinical applications. Wang et al. [147] demonstrated that miR-122 acts as a tumor suppressor and plays an important role in inhibiting tumorigenesis through targeting IGF1R and regulating PI3K/Akt/mTOR/p70S6K pathway.

In breast cancer specimens, miR-497 expression pattern was negatively correlated with pathological stage, lymphatic metastasis, tumor size, and HER-2, and no correlation was found between miR-497 and ER, PR and p53 status. The overexpression of miR-497 results in downregulation of Bcl-w (antiapoptotic member of the Bcl-2 family), causing cellular growth inhibition and apoptotic enhancement, as well as G0/G1 phase arrest, acting like a tumor suppressor. Thus, breast cancer patients with elevated expression of miR-497 have better prognosis, and this marker may turn out to be a new prognostic marker [148].

Genome-wide analyses have identified deregulated miRNA expression in human malignancies [149] and a potential dual role in tumor formation, highlighting that miRNAs can modulate oncogenic or tumor suppressor pathways, including *p53, c-MYC, RAS,* and *BCR-ABL*, while expression of miRNAs themselves can be regulated by oncogenes or tumor suppressors.

However, miRNA can be prejudicial to breast cancer patient, contributing to tumor development. For instance, miR-373 and miR-520c stimulate cancer cell migration and invasion in vitro and in vivo [150]. Many studies have demonstrated the potential of miRNAs, as regulators, and they may serve as novel diagnostic and prognostic candidates and potential therapeutic targets.

5. Conclusion

Breast cancer is the second leading cause of women mortality and morbidity worldwide and this cancer represents one of the most privileged malignancy regarding the use of

markers with predictive values, but especially two therapeutic strategies of great clinical relevance are known and applied in patient routine, Tamoxifen and Trastuzumab. Otherwise, many women are still diagnosed in 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. In this context, some of the molecules discussed in this review provided strong evidence that evaluation and application of these breast cancer markers will play a significant role in more effective and targeted therapies, with lower toxicity to patients.

Conflict of Interests

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

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CCR2 gene: a possible marker in HER2+ breast cancer molecular subtype

Abstract

Breast cancer (BC) is one of the most common cancers in women. Many tumor cell types can express chemokines and chemokine receptors, and for this reason, these molecules can affects both tumor progression and anti-tumor immune response. Polymorphisms of several receptors were found to be risk factors for cancer development. The purpose of this study was to investigate the impact of CCR2-V64I and CCR5-Δ32 polymorphisms on susceptibility and clinical outcomes of BC. The genotyping was done by PCR based methodologies in 118 histological confirmed patients and in 180 controls neoplasia free. No significant associations between CCR5-Δ32 and CCR2-V64I polymorphisms and BC susceptibility (CCR5-Δ32: OR=1.04; CI95%=0.60-1.81; CCR2-V64I: OR=1.32; CI95%=0.57-3.06) or clinical outcome (tumor size, lymph nodes commitment and/or distant metastasis, staging and nuclear grade) were found. However, the analysis that sought a correlation between the genetic variants and the development of subtypes of breast tumors, showed statistic significance between the CCR2-V64I allelic variant and the HER2 positive samples of BC (p=0.026). This study shows that CCR5-Δ32 and CCR2-V64I polymorphisms not confers susceptibility and also are not correlated with clinical outcomes in a Brazilian population with breast cancer. Therefore, we found a positive correlation between a mutation in *CCR2* gene and a worst prognostic subgroup of breast tumor.

Key-words: CCR2, CCR5, HER2, polymorphism, breast cancer, clinical outcome, molecular subtypes.

Introduction

Despite advances in diagnosis and treatment of human malignancy, cancer remains amongst the leading causes of morbidity and mortality worldwide. Breast cancer is the second most common cancer in the world and, by far, the most frequent cancer among women with an estimated 1.7 million new cancer cases diagnosed in 2012 (25% of all cancers). This disease is also the most common cause of cancer death among women, accounting for 522 000 deaths in 2012 (FERLAY *et al*. 2013).

Breast cancer is a heterogeneous and phenotypically diverse disease. It is composed of several biologic subtypes that have distinct behaviors and responses to therapy. Among these subtypes, 20 to 30% have the oncogene HER2 amplification, which is considered a marker of poor prognosis, once its overexpression is associated with an aggressive phenotype of tumor cells, resistance to anti-hormonal and cytotoxic therapies and low overall survival (CITRI; SKARIA; YARDEN, 2003). The HER2 is a transmembrane tyrosine kinase receptor belonging to a family of epidermal growth factor receptors structurally related to epidermal growth factor receptor (EGFR), encoded by *ERBB2/HER2* oncogene located on the chromosome 17q21 (YAMAMOTO et al., 1986). The homodimerization or heterodimerization of HER family receptors activates intracellular tyrosine kinase domain which promotes the autophosphorylation of tyrosine residues of cytoplasmatic tail and thus triggers pathways that results in survival and cellular proliferation (CITRI; SKARIA; YARDEN, 2003).

There is lot evidence that tumors can elicit an immune response in its microenvironment, which generally, is considered ineffective to destroy cancer cells. However, in the last years evidence has emerged demonstrating the importance of immune cells infiltration, such as lymphocyte and tumor-associated macrophages (TAM), in the clinical evolution of many cancer types, through non immune (mostly proangiogenic) and immune mechanisms (OSTRAND-ROSENBERG; SINHA, 2009; CRUZ-MERINO *et al.*, 2013).

The activation and recruitment of leucocytes is regulated by chemotactic and proinflammatory chemokines and their receptors. CC chemokine receptors, CCR2 and CCR5 are G protein coupled receptors that bind chemokines, such as MCP-1 (monocyte chemoattractant protein-1), RANTES (regulated on activation, normal T cell expressed and secreted) and MIP-1 (macrophage inflammatory protein-1) (MURPHY, 1996).

CCR2 is mainly expressed by monocytes/macrophages, basophils, mast cells, T lymphocytes, NK cells, and dendritic cells (CHARO *et al*., 1994; POLENTARUTTI *et al*., 1997; SANDERS *et al*., 2000). It is known that polymorphisms of several receptors were found to be risk factors for development of different types of cancer. In this context, the CCR2-V64I (rs1799864) polymorphism consists in a substitution from guanine to adenine at position 190 of this gene that results in a substitution from valine to isoleucine at position 64 in the CCR2 protein (SMITH *et al*., 1997; KOSTRIKIS *et al.*, 1998), which results in the enhanced gene expression and prolonged half-life of CCR2A isoform (NAKAYAMA *et al.*, 2004).

CCR5 is present in certain cell types, specifically, lymphocytes, dendritic cells, and macrophages (LOETSCHER *et al*., 1998). The CCR5-Δ32 (rs333) polymorphism corresponds to a 32-bp deletion that occur at a site of a repeat motif in the *CCR5* gene and results in a frame shift in the coding sequence producing a defective protein, which is not expressed on the cell surface (MCNICHOLL *et al*., 1997).

Polymorphisms in *CCR2* and *CCR5* genes were found to be risk factors for the development of different types of cancer, such as metastatic melanoma, oral and bladder cancer (UGUREL *et al.*, 2008; CHEN, M. K. *et al*., 2011; KUCUKGERGIN *et al*., 2012). However, also was found, that polymorphism in *CCR2* gene also confer significant protection to breast cancer, in Greek women (ZAFIROPOULOS *et al*., 2004).

In light of these findings, we aimed to determine whether genetic variants in CCR2-V64I and CCR5-Δ32 are associates with breast cancer susceptibility and/or correlated with clinical outcome and breast cancer subtypes in a Brazilian population in the South region of the country.

Material and Methods

Human subjects

The protocol was approved by the Institutional Human Research Ethics Committee of the State University of Londrina, Paraná, Brazil (CEP/UEL 189/2013, CAAE 17123113400005231). The individuals were invited to participate, informed in detail regarding the research and voluntary written consent term was obtained. 5 ml of peripheral blood was collected with ethylenediaminetetra acetic (EDTA) from 118 breast cancer patients, aged 23-84 years, attended in the Cancer Hospital of Londrina (Hospital do Câncer

de Londrina) and 180 healthy women neoplasia free, from Blood Center of the Northern Region of Paraná (Hemocentro da Região Norte do Paraná).

Clinical outcomes and subtypes of breast cancer

The data relating to clinic pathological parameters and molecular subtypes of breast cancer were kindly provided by the Cancer Hospital of Londrina (Londrina, Paraná, Brazil). The clicopathological parameters assessed include: tumor size, lymph nodes commitment and/or distant metastasis, nuclear grade and clinical staging, which was determined according to the Union of International Control of Cancer classification criteria (SOBIN; GOSPODAROWICZ; WITTEKIND, 2009). The breast cancer subtypes studied in this sample were: Luminals A and B (positive for estrogen and/or progesterone receptors), HER2+ subtype (positive for the HER2 oncogene overexpression) and the triple negative (negative for the hormone receptors status and HER2 overexpression), determined according to the immunohistochemical analysis also available in the medical records provided by Cancer Hospital of Londrina.

Genomic DNA extraction

Genomic DNA was extracted from whole blood by Biopur Mini Spin Plus Kit (Biometrix Diagnóstica, Curitiba, Paraná, Brazil), according to the manufacturer's instructions. DNA was ressuspended in 50μL of elution buffer and quantified by NanoDrop 2000c®Spectrophotometer (Thermo Scientific, Wilmington, Delaware, USA) at a wavelength of 260/280 nm. Final preparation was stored at -20ºC and used as templates in polymerase chain reactions (PCR).

CCR2-V64I and CCR5-Δ32 genotyping

Genotyping of CCR5-Δ32 (rs333) was determined by conventional PCR, using specific primers obtained from Martinson et al. (1997), and the CCR2-V64I (rs1799864) was genotyped by PCR-RFLP using specific primers designed by Sezgin et al. (2011). The samples were amplified using approximately 100 ng of genomic DNA and the buffer kit plus 1.25units/reaction Taq polymerase (Invitrogen^{TM}, Carlsbad, California, USA) in a Master cycler Gradient (Eppendorf, Hamburg,Germany). In each PCR, negative control, without DNA, were employed to make sure that no contaminants were introduced in the initial PCR.

CCR5-Δ32 PCR conditions were: denaturation at 94˚C for 5 min, 35 cycles of 1 min at 94˚C, 1 min at 58˚C and 1 min at 72˚C, and 10 min of elongation at 72˚C. And the CCR2-

V64I PCR conditions were: denaturation at 94ºC for 5 min, 40 cycles of 30 sec at 94ºC, 30 sec at 55ºC and 30sec at 72ºC, and 5 min of elongation at 72ºC. PCR product (2µl) was digested for 4 hours at 60ºC with 5U/reaction of BsaBI restriction endonuclease (New England Biolabs, Beverly, Massachusetts, USA). The PCR and digested products were analyzed on polyacrylamide gel (10%), stained with silver nitrate ($AgNO_3$). The specific primers, expected products of PCR and enzymatic restriction are given in Table 1.

Statistical analysis

An estimative of the relative risk at 95% confidence intervals (CI) was calculated as the odds ratio (OR) to the case-control association study, between patients and controls, using a 2 x 2 contingency tables, with the considered wild type genotype as reference (OR=1.0). The correlations between the polymorphisms and clinical parameters or breast cancer subtypes were performed by Spearman's Rho test (SPSS inc., Chicago, Illinois, USA). Values of $p<0.05$ were considered statistically significant.

Table 1. Primers, expected PCR and restriction products of CCR2-V64I and CCR5-Δ32 polymorphisms.

	Primers	PCR Products	Restriction enzyme	Restriction Products
CCR2-V64I (rs1799864)	Sense: 5'-CAT TGC AAT CCC AAA GAC CCA CTC-3' Anti-sense: 5'-TTG GTT TTG TGG GCA ACA TGA TGG-3'	173bp	BsaBI	149bp 24bp
CCR5-Δ32 (rs333)	Sense: 5'-ACC AGA TCT CAA AAA GAA-3' Anti-sense: 5'-CAT GAT GGT GAA GAT AAG CCT CA-3'	225bp 193bp	-	-

Results

Genotype frequency distributions were in agreement with the Hardy-Weinberg equilibrium in all sample groups ($p>0.05$). Although for some patients specific clinic pathological characteristics were not available, we observed that of 93.9% had ductal carcinoma, 4.3% lobular carcinoma and 1.7% rare subtypes of breast cancer (n = 116), 87.3% of the patients had nuclear grade in stages II or III (n = 102), 47.4% had lymph node involvement and/or metastasis (n = 95) and the mean tumor size was 2.9 cm (n = 105).

Eletrophoretic profile for CCR2-V64I and CCR5-Δ32 polymorphisms are demonstrated in Figure 1. The genotype distribution and allele frequencies of the CCR2-V64I and CCR5-Δ32 polymorphisms are shown in Table 2. In this study we did not found any association between the polymorphisms analyzed and breast cancer susceptibility (CCR2-

V64I: OR=1.32; CI 95%=0.57-3.06; CCR5-Δ32: OR=1.04; CI 95%=0.60-1.81). The case control association study taking into account the combination of genotypes considered of risk (CCR5-Δ32 + CCR2-V64I) was not possible due to the low number of individuals that were simultaneously heterozygous (only one patient and one control) or homozygous mutants (any patient and control) in this sample.

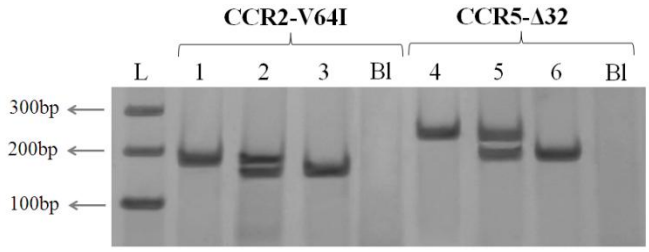

Figure 1. Eletrophoretic profile of CCR2-V64I (rs1799864) and CCR5-Δ32 (rs333) polymorphisms. Polyacrylamide gel 10% stained with silver nitrate. Lane 1, wild-type homozygous genotype (G/G) yield 173-bp; Lane 2, heterozygous genotype (G/A) 173-bp and 149-bp products; Lane 3, mutated homozygous genotype (A/A) yield a 149-bp product; Lane 4, wild-type homozygous genotype (WT/WT) yield 225-bp; Lane 5, heterozygous genotype (WT/Δ32) 225-bp and 193-bp products; Lane 6, mutated homozygous genotype (Δ32/Δ32) yielded a 193-bp product. L: 100bp molecular weight marker (Invitrogen™, Carlsbad, California, USA); Bl: Blanc reaction or negative control.

Table 2. Allelic and genotypic frequencies of CCR2-V64I (rs1799864) and CCR5-Δ32 (rs333) polymorphisms.

		Controls n = 180	**Patients n = 118**
CCR2-V64I (rs1799864)	GG	140 (77.78%)	91 (77.12%)
	GA	37 (20.55%)	25 (21.19%)
	AA	3 (1.67%)	2 (1.69%)
	G allele	88%	88%
	A allele	12%	12%
CCR5-Δ32 (rs333)	WT/WT	167 (92.78%)	107 (90.68%
	WT/Δ/32	12 (6.67%)	11 (9.32%)
	Δ32/Δ32	1 (0.55%)	0 (0%)
	WT allele	96%	95%
	Δ32 allele	4%	5%

The analysis using clinical parameters and subtypes of breast cancer (Table 3) found no significative correlation with tumor size, lymph nodes commitment and/or distant metastasis, staging, nuclear grade and with triple-negative and hormonal receptor subtypes. There was also no significant correlation between the CCR5-Δ32 and overexpression of HER2, but was found significative correlation between this breast cancer subtype and the CCR2-V64I allelic variant (p=0.026).

Table 3. Correlation analysis of CCR2-V64I (rs1799864) and CCR5-Δ32 (rs333) polymorphisms with clinical outcome or subtypes of breast cancer.

		Number of individuals n(%)	CCR2 V64I genotype		CCR5Δ32 genotype	
			GG n (%)	GA+AA n (%)	WT/WT n (%)	WT/Δ32 + Δ32/Δ32 n (%)
Tumor size (n = 105)	0 - 1.5cm	17 (16.19%)	13 (12.38%)	4 (3.81%)	16 (15.24%)	1 (0.95%)
	1.5 - 3.0cm	61 (58.10%)	48 (45.71%)	13 (12.38%)	54 (51.43%)	7 (6.66%)
	> 3.0cm	27(25.71%)	20 (19.05%)	7 (6.67%)	24 (22.86%)	3 (2.86%)
	p value		*p* = 0.784		*p* = 0.655	
Lymph nodes commitment and/or metastasis (n = 95)	Present	45 (47.37%)	35 (36.84%)	10 (10.53%)	42 (44.21%)	3 (3.16%)
	Absent	50 (52.63%)	40 (42.10%)	10 (10.53%)	44 (46.32%)	6 (6.31%)
	p value		*p*=0.631		*p*=0.622	
Staging (n = 115)	I	20 (17.39%)	15 (13.04%)	5 (4.35%)	19 (16.52%)	1 (0.87%)
	II	52 (45.22%)	41 (35.65%)	11 (9.56%)	48 (41.74%)	4 (3.48%)
	III	35 (30.43%)	27 (23.48%)	8 (6.96%)	29 (25.22%)	6 (5.22%)
	IV	8 (6.96%)	6 (5.22%)	2 (1.74%)	8 (6.95%)	0 (0.00%)
	p value		*p*=0.985		*p*=0.283	
Nuclear grade (n = 102)	I	13 (12.74%)	11 (10.79%)	2 (1.96%)	12 (11.77%)	1 (0.98%)
	II	42 (41.18%)	34 (33.33%)	8 (7.84%)	36 (35.29%)	6 (5.88%)
	III	47 (46.08%)	35 (34.31%)	12 (11.77%)	45 (44.12%)	2 (1.96%)
	p value		*p*=0.649		*p*=0.252	
Hormonal receptor (n = 111)	Positive	85 (76.58%)	66 (59.46%)	19 (17.12%)	76 (68.47%)	9 (8.11%)
	Negative	26 (23.42%)	20 (18.02%)	6 (5.40%)	25 (22.52%)	1 (0.90%)
	p value		*p*=0.298		*p*=0.939	
HER2 (n = 99)	Positive	22 (22.22%)	13 (13.13%)	9 (9.09%)	21 (21.21%)	1 (1.01%)
	Negative	77 (77.78%)	63 (63.64%)	14 (14.14%)	68 (68.69%)	9 (9.09%)
	p value		*p*=0.026*		*p*=0.332	
Triple negative (n = 99)	Triple negative	17 (17.17%)	14 (14.14%)	3 (3.03%)	16 (16.16%)	1 (1.01%)
	Other subtypes	82 (82.83%)	62 (62.63%)	20 (20.20%)	73 (73.74%)	9 (9.09%)
	p value		*p*=0.554		*p*=0.531	

n – Number of subjects; Spearman's Rho test *Value of $p<0.05$ was considered statistically significant.

Discussion

Chemokines and their receptors play a pivotal role in the development of different types of cancer (BALKWILL, 2004). Some studies have shown that CCL2 and CCL5 expressions are higher in breast cancer tissue than in corresponding normal tissue (ZHANG *et al*., 2009; FANG *et al*., 2012), highlighting the importance of their respectively receptors in the development of this disease. The CCL2/CCR2 signaling has been implicated in the breast cancer cell motility (FANG *et al*., 2012) and the macrophages recruitment (QIAN *et al*., 2012). Likewise the CCL5/CCR5 axis also showed correlation with metastatic phenotype, but only in basal and HER2 positive breast cancer (VELASCO-VELAZQUEZ; PESTELL, 2013).

Nakayama et al. (2004) reported that the level of cell surface expression of mutated CCR2A was significantly higher than that of CCR2A without the substitution. Furthermore, when co-expressed with CCR5, the mutated CCR2A interfered more severely with cell surface expression of CCR5 than did wild-type CCR2A, suggesting that CCR2A binds to CCR5 in the cytoplasm and down-modulates its surface expression. Therefore, although this polymorphism could favor the CCL2/CCR2 axis due to increased cell surface expression of CCR2A, it could down-modulate the CCL5/CCR5 axis by decrease the CCR5 expression on cell surface.

It is known that polymorphisms of several receptors were found to be risk factors for development of different types of cancer. In this context, the present study investigated the CCR2-V64I and CCR5-Δ32 genetic polymorphisms on breast cancer in a Brazilian population and also whether they could be potential markers of clinical outcome for this disease.

Attar *et al*. (2010) demonstrated that the mutated allele, of CCR2-V64I polymorphism, is associated with increased risk for endometrial cancer in Turkish population. Furthermore, some studies have shown that in Taiwanese population, this mutated allele also is associated with increased risk for hepatocellular (YEH *et al*., 2010) and oral cancer (CHEN *et al*., 2011). Both polymorphisms, CCR2-V64I and CCR5-Δ32, are associates with prostate cancer risk, in a Turkish population (KUCUKGERGIN *et al*., 2012). While, are not associate with cervical neoplasia in Swedish population (ZHENG et al., 2006).

In this study, no association between the CCR2-V64I and CCR5-Δ32 variants and breast cancer susceptibility was verified (CCR2-V64I: OR=1.32; CI 95%=0.57-3.06; CCR5-Δ32: OR=1.04; CI 95%=0.60-1.81). These results are in accordance with works developed by Guleria *et al*. (2012), Zafiropoulos *et al.* (2004), Degerli, Yilmaz and Bardakci (2005) and

Aoki *et al.* (2009), who also no found any association between the Δ32 allele and breast cancer in Indian, Greek, Turkish and Brazilian populations, respectively.

However, regarding the CCR2-V64I polymorphism result, our work is in contrast with Zafiropoulos *et al.* (2004), which studied Greek population and found association between this polymorphism and breast cancer susceptibility. According these authors, genotype grouping revealed significant breast cancer protection for the mutated allele (OR = 0.53; CI 95% = 0.33 - 0.84) and the heterozygote genotype (OR = 0.54; CI 95% = 0.33 - 0.89).

CCR5-Δ32 frequency is not distributed equally among the world's population. Genetic studies have shown high frequency of Δ32 allele (approximately 10%) in Caucasians; however, it has not been found in African, Native American and Eastern Asian populations. In contrast to CCR5-Δ32, the mutant allele of CCR2-V64I polymorphism is more common in African–American and Asian populations than in northern Europe Caucasians (GHARAGOZLOO *et al*., 2005). In this work, was genotyped a sample group from Brazilian population, which suffers a considerable miscegenation, and was found a low frequency of Δ32 (*CCR5* gene) and A (*CCR2* gene) alleles. The absence of association between the studied polymorphisms and breast cancer susceptibility can be explained by low frequency of these mutant alleles. Thus, there may be a need to increase the sample size to confirm or not our findings.

According to our knowledge, were no found in the literature correlation analysis between clinical outcomes and CCR2 and/or CCR5 polymorphisms. Therefore, this study found no significant correlations for tumor size, lymph nodes commitment and/or distant metastasis, staging and nuclear grade (Table 3). We also conducted correlation analysis separating the sample of patients for the most common breast cancer subtypes, characterized by immunohistochemical profile: hormonal receptors positive, HER2 positive and triple negative. There were no significative correlations between genetic variants and triple-negative or hormonal receptor (Table 3).

Regarding the CCR5-Δ32 polymorphism, we expected a protective effect of allelic variant against metastasis since the 32-bp deletion produce a defective protein that is not expressed on the cell surface, which could lead to decreased invasive potential of tumor cells. But, this correlation was not found, maybe this can be explained because in our study the majority of the samples were HER2 negative (77.78%) and according a work developed by Velasco-Velazquez *et al.* (2012) the CCL5/CCR5 signaling seems be preferentially activated during development of specific breast cancer subtypes, like HER2 positive. Although there was no significant correlation between the CCR5-Δ32 and overexpression of HER2,

interestingly it was found a significance between this clinical parameter and the CCR2-V64I polymorphism (p=0.026). Overexpression of HER2 oncogene, either through gene amplification or through transcriptional deregulation is seen in approximately 25-30% of breast and confers worse biological behavior (SLAMON *et al.*, 1989). Since the HER2 confers a poor prognosis in breast cancer and given the correlation between this tumor subgroup and the CCR2 polymorphism in our study, the *CCR2* gene may be a promising marker for this neoplasia. However, there may be necessary confirm this hypothesis, in future studies, with samples exclusively of HER2 + subtype.

It is known that HER2-overexpression was first shown to activate NF-κB over a decade ago (GALANG *et al.*, 1996), however, the role that NF-κB plays in development and progression of HER2-overexpressing in breast cancer is still poorly understood. Additionally, the pathway leading to NF-κB activation downstream of HER2 is not well characterized (MERKHOFER; COGSWELL; BALDWIN, 2010). NF-κB is an important transcription factor that has been shown to be involved in expression of genes implicated in key cellular processes including innate and adaptive immunity (BONIZZI; KARIN, 2004) as well as being activated in different cancers, including breast cancer (BELGHISE; SONENSHEIN, 2007).

The HER2-overexpression in breast carcinoma is controlled not only by the degree of amplification of the gene but also at the level of gene transcription (BOSHER; WILLIAMS; HURST, 1995). Although the context of the study developed by Jimenez et al. (2010) differently from the present work, these authors showed that CCL2 acts on its receptor CCR2 and can induce the translocation of the transcription factor NF-κB into the nucleus. This transcription factor is known to bind directly to the HER2 promoter, resulting in HER2-overexpression (CAO et al., 2009). Thus, the CCL2/CCR2 signaling could to contribute to HER2-overexpression.

In summary, chemokines and their receptors have been implicated in breast cancer and our dates indicate that the *CCR2* gene might be a possible marker in the HER2+ subgroup. Further studies involving a large sample size are necessary to verify this correlation and finally establish a possible mechanism between CCR2 receptor and HER2-overexpressing in breast tumors progression.

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Conclusão

- Não houve associação significativa entre os polimorfismos CCR2-V64I e CCR5-Δ32 e a susceptibilidade aumentada ao desenvolvimento do câncer de mama.

- Não houve correlação significativa entre os polimorfismos genéticos de *CCR2* ou *CCR5* com os parâmetros clínicopatológicos avaliados: tamanho do tumor, acometimento de linfonodos e/ou metástase à distância, estadiamento clínico e grau nuclear.

- Também não observamos correlações significativas entre o polimorfismo CCR5-Δ32 e subtipos do câncer de mama classificados segundo o perfil de imunohistoquímica.

- O polimorfismo CCR2-V64I não se mostrou correlacionado aos subtipos tumorais: triplo-negativo e receptores hormonais positivos.

- Entretanto, houve correlação entre o polimorfismo CCR2-V64I e o subtipo tumoral que contém a superexpressão do oncogene HER2, indicando que o gene *CCR2* pode ser um marcador prognóstico promissor neste subtipo específico do câncer de mama.

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Anexos

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(o) 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 nehuma compemsação financeira adicional. A coordenadora do projeto é a Profª. Drª Maria Angelica Ehara Watanabe, que pode ser encontrada no endereço: Rod. Celso Garcia cid, 445, Departamento de Ciências Patológicas, Centro de Ciências Biológicas, Universidade Estadual de Londrina, CEP: 86051-970, Tel / Fax: (43) 3371-5629, 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 esclarecido pelo(s) pesquisador(es) dos objetivos e procedimentos da pesquisa de maneira clara e detalhada, e esclareci minhas dúvidas. Concordo em participar voluntariamente desse estudo sendo **permitindo a colheita do sangue do meu filho(a)** sendo que poderei tirar meu consentimento a qualquer momento, antes ou durante o mesmo, sem penalidades ou prejuízos no meu atendimento neste projeto.

Londrina, ____ de _____, 20 ___.

Assinatura do responsável (ou representante legal): _____

Hospital do Câncer de Londrina

DECLARAÇÃO

À Profa. Dra. Maria Angélica Ehara Watanabe
Depto. Ciências Patológicas

Prezada,

O Instituto de Câncer de Londrina declara para os devidos fins, que é colaborador no Projeto de Pesquisa sob o tema "Estudo de marcadores genéticos, epigenéticos, moleculares e imunológicos em câncer", que se encontra em fase de submissão e aprovação pelo Comitê de Ética em Pesquisa da UEL.

Neste Projeto será realizada a obtenção de amostra de sangue, tecido e blocos de parafina de pacientes portadores de tumores mamários, de intestino e colon bem como a realização de consultas aos prontuários, na busca por informações clínicas e histopatológicas que possam ser correlacionadas aos dados obtidos com as pesquisas básicas. Nenhuma intervenção terapêutica será realizada pelos pesquisadores e todos os sujeitos de pesquisa assinarão um Termo de Consentimento Livre e Esclarecido antes de qualquer procedimento do Estudo.

O referido estudo terá início somente após parecer favorável do Comitê de Ética em Pesquisa.

Sendo o que tínhamos, agradecemos

08/04/13

Dr. Jesus Roberto Ceribelli
Diretor Clínico

Universidade Estadual de Londrina

PARANÁ

HOSPITAL UNIVERSITÁRIO
DIRETORIA SUPERINTENDENTE

PARECER PROCESSO 12901 . 2013 . 87

À Pesquisadora

Maria Angélica Ehara Watanabe

Considerando o Projeto de Pesquisa com o título "ESTUDO DE MARCADORES GENÉTICOS EPIGENÉTICOS MOLECULARES E IMUNOLÓGICOS EM CÂNCER" apresentado a esse Hospital Universitário, estando vinculado ao Programa de Pós-Graduação em Patologia Experimental - Centro de Ciências Biológicas/UEL;

Considerando o parecer favorável apresentado nas instâncias administrativas que envolvem a realização do estudo;

Considerando que o projeto deverá ser analisado pelo Comitê de Ética em Pesquisa do HU/UEL para posterior operacionalização, atendendo a Resolução 196/96 do Conselho Nacional de Pesquisa;

Vimos informar que **somos de parecer favorável à sua realização, resguardando-se o atendimento da legislação vigente.**

Solicitamos que, tão logo o Comitê de Ética emita parecer, que essa Diretoria Superintendente seja notificada, para os procedimentos cabíveis relacionados à documentação da pesquisa

Solicitamos também que, uma vez realizado o estudo, uma cópia seja apresentada a esta Diretoria Superintendente, para ciência e divulgação.

Em 29/05/2013.

Profa. Dra. Margarida de Fátima Fernandes Carvalho
Diretora Superintendente do HU

Plataforma Brasil MINISTÉRIO DA SAÚDE - Conselho Nacional de Saúde - Comissão Nacional de Ética em Pesquisa – CONEP

FOLHA DE ROSTO PARA PESQUISA ENVOLVENDO SERES HUMANOS

1. Projeto de Pesquisa:	2. Número de Sujeitos de Pesquisa:
Estudo de marcadores genéticos, epigenéticos, moleculares e imunológicos em câncer	800

3. Área Temática:

4. Área do Conhecimento:
Grande Área 2. Ciências Biológicas

PESQUISADOR RESPONSÁVEL

5. Nome:
Maria Angelica Ehara Watanabe

6. CPF:	7. Endereço (Rua, n.º):		
364.985.139-34	CELSO GARCIA CID 900 JARDIM PORTAL DE VERSALHES 1 LONDRINA PARANA 86057970		
8. Nacionalidade:	9. Telefone:	10. Outro Telefone:	11. Email:
BRASILEIRA	(43) 3371-5629		maewatuel@gmail.com

12. Cargo:

Termo de Compromisso: Declaro que conheço e cumprirei os requisitos da Resolução CNS 196/96 e suas complementares. Comprometo-me a utilizar os materiais e dados coletados exclusivamente para os fins previstos no protocolo e a publicar os resultados sejam eles favoráveis ou não. Aceito as responsabilidades pela condução científica do projeto acima. Tenho ciência que essa folha será anexada ao projeto devidamente assinada por todos os responsáveis e fará parte integrante da documentação do mesmo.

Data: 15 / Maio / 2013

Profª. Drª. Maria Angelica Ehara Watanabe
CRBM 2361
Universidade Estadual de Londrina

Assinatura

INSTITUIÇÃO PROPONENTE

13. Nome:	14. CNPJ:	15. Unidade/Órgão:
Universidade Estadual de Londrina - UEL	78.640.489/0001-53	Programa de PG em Patologia Experimental
16. Telefone:	**17. Outro Telefone:**	
(43) 3371-4387		

Termo de Compromisso (do responsável pela instituição): Declaro que conheço e cumprirei os requisitos da Resolução CNS 196/96 e suas Complementares e como esta instituição tem condições para o desenvolvimento deste projeto, autorizo sua execução.

Responsável: _____ CPF: 51212927 8-34

Cargo/Função: _____

Data: 15 / 05 / 2013

Prof. Dr. Rubens Cecchini
Coord. do Programa de Pós-Graduação
em Patologia Experimental

PATROCINADOR PRINCIPAL

18. Nome:	19. Telefone:	20. Outro Telefone:
6088 Fundação Araucária	(41) 3271-4873	

Termo de Compromisso: Declaro que conheço e cumprirei os requisitos da Resolução CNS 196/96 e suas complementares. Comprometo-me a utilizar os materiais e dados coletados exclusivamente para os fins previstos no protocolo e a publicar os resultados sejam eles favoráveis ou não. Aceito as responsabilidades pela condução científica do projeto acima.

Nome: _____ CPF: _____

Cargo/Função: _____ Email: _____

Data: _____ / _____ / _____

_____
Assinatura

Universidade Estadual de Londrina

PARANÁ
GOVERNO DO ESTADO

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

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

Prezado(a) Senhor(a):

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

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

Situação do Projeto: **Aprovado**

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

Londrina, 30 de setembro de 2013.

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

Comitê de Ética em Pesquisa Envolvendo Seres Humanos - UEL