MARLA KARINE AMARANTE

RECEPTOR TOLL-LIKE 3:

POSSÍVEL IMUNOMODULAÇÃO ATRAVÉS DE RNA E IMPLICAÇÕES NO MICROAMBIENTE DO CÂNCER DE MAMA

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Orientadora: Profª. Drª. Maria Angelica Ehara Watanabe.

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RESUMO

A investigação dentro da imunologia envolvendo RNA tem sido amplamente explorada com o reconhecimento da existência do receptor Toll like 3 (TLR3) o qual interage com RNAs de origem viral. TLR3 reconhece RNA dupla fita, como por exemplo RNA poly (I:C), um análogo sintético de RNA dupla-fita (RNAds) viral e sua estimulação resulta na liberação de citocinas e quimiocinas proinflamatórias. É conhecido que RNAds, além de desencadear a sinalização em cascata para produção de citocinas, como interferons (INFs), também ativa enzimas induzidas por INF, incluindo proteína quinase dependente de RNA (PKR). No presente estudo foi investigada a expressão relativa de RNAm para TLR3, CXCR4, IFNγ e PKR em culturas de células sensibilizadas com poli (I:C) e RNA endógeno proveniente de células mononucleadas do sangue periférico (PBMC) humano. Não foi observado efeito citotóxico e aumento na proliferação de células $CD3^+$, $CD4^+$ e $CD8^+$. Houve aumento da expressão relativa de RNAm de TLR3, IFNγ, CXCR4 e PKR em cultura de PBMC com poli (I:C). Embora tenha sido observado que TLR3 reconhece RNAds sintético e ativa genes que aumentam as citocinas inflamatórias importantes para a interação das células imunes, a função fisiológica do TLR3 permanece a ser elucidada. Interessantemente, a cultura de células sensibilizadas com RNA endógeno autólogo apresentou diminuição da expressão de RNAm de TLR3, IFNγ, CXCR4 e PKR. É possível que os RNAs endógenos como microRNAs e RNAs longos, não codificadores, através de suas complexas estruturas secundárias participem na regulação da expressão gênica independente do TLR3. É conhecido que TLRs são expressos não somente pelas células do sistema immune, mas também por células tumorais e que IFNγ pode ser uma citocina chave na resposta imune antitumoral. A próxima etapa do presente trabalho foi investigar a expressão de RNAm de TLR3, IFNγ e CXCR4 por PCR em tempo real, em tecido mamário tumoral das pacientes com câncer de mama (carcinoma ductal) comparado com tecido mamário saudável. Embora não tenha sido verificada diferença significante entre o tecido mamário saudável e tecido mamário tumoral quanto à expressão de TLR3, houve aumento estatisticamente significante da expressão do TLR3 no tecido mamário tumoral de pacientes sem acometimento de linfonodos. Foi verificada correlação positiva da expressão de RNAm para TLR3 e IFNγ e também para TLR3 e CXCR4, portanto é possível que estas moléculas tenham implicações na patogênese do câncer. A etiologia do câncer de mama tem sido muito discutida, variáveis como predisposição genética, idade e ambiente são comprovadamente fatores de risco, porem não são absolutos e únicos. Alguns vírus, devido a sua complexidade na estrutura e mecanismo de ação, são considerados agentes etiológicos de algumas neoplasias, como câncer de colo de útero, câncer de mama, linfoma e leucemias. O vírus do tumor mamário de camundongos (MMTV) tem sido sugerido como candidato a vírus causador de câncer de mama. É provável que algumas pacientes com câncer de mama possam apresentar envolvimento viral e que o TLR3 possa ser um receptor envolvido na resposta imune como também num

processo carcinogênico na mama. A participação do RNA na patogênese da doença e suas implicações na modulação da resposta imune como também na terapêutica é um tema a ser elucidado.

Palavras-chave: TLR3. IFNγ. CXCR4. PKR. RNA. MMTV.

AMARANTE, M. K. **Toll-like receptor 3:** possible immunomodulation by RNA and implications in the microenvironment of breast cancer. 2011. 95f. Thesis (Doctorate in Experimental Pathology) – Department of Pathological Sciences, State University of Londrina, Londrina, 2011.

ABSTRACT

The research of immunology involving RNA has been widely exploited with the recognition of Toll like receptor 3 (TLR3) which interacts with viral RNAs. TLR3 recognizes double stranded RNA such as RNA poly (I: C), a synthetic analogue of double-stranded RNA (RNAds) virus and its stimulation results in release of proinflammatory cytokines and chemokines. It is known that RNAds, besides triggerring the cascade signalization for the production of cytokines such as interferons (INFs), also activates enzymes induced by INF, including RNA-dependent protein kinase (PKR). This study investigated the relative expression of mRNA for TLR3, CXCR4, IFNg and PKR in sensitized cultured cells with poly (I: C) and endogenous RNA from peripheral blood mononuclear cells (PBMC) human. No cytotoxic effect or increased proliferation of $CD3^+$, $CD4^+$ and $CD8^+$ were observed. There was an increased mRNA expression for TLR3, IFNγ, CXCR4 and PKR in cultured PBMC with poly (I: C). Although it was observed that TLR3 recognizes dsRNA synthetic and activates genes that increase the inflammatory cytokines important for the interaction of immune cells, the physiological function of TLR3 remains to be elucidated. Interestingly, the cultured cells sensitized with endogenous RNA exhibited reduced mRNA expression of TLR3, INFγ, CXCR4 and PKR. It is possible that endogenous RNAs such as microRNAs and long RNAs, not coders, through their complex secondary structures are involved in regulating gene expression independent of TLR3. It is known that TLRs are expressed not only by cells of the immune system but also by tumor cells and that IFNγ can be a key cytokine in anti-tumor immune mechanism. The next step of this study was to investigate mRNA expression of TLR3, IFNγ and CXCR4 by real time PCR in breast tumor tissue of patients with breast cancer (ductal carcinoma) compared with normal breast tissue. Although no significant difference was found between normal tissue and breast in the expression of TLR3, there was a statistically significant increase in the expression of TLR3 in tumor breast tissue of patients without lymph node involvement. It was found positive correlation between mRNA increase for TLR3 e IFNγ and too TLR3 and CXCR4, so it is possible to involvement the increase of mRNA expression for TLR3 and IFNγ that these molecules have implications in the pathogenesis of cancer. The etiology of breast cancer has been much discussed, variables such as genetic predisposition, age and environment are proved risk factors, but are not absolute and unique. Some viruses, due to its complexity in structure and mechanism of action, are considered etiologic agents of some malignancies such as cervical cancer, breast cancer, lymphoma and leukemia. The mammary tumor virus of mice (MMTV) has been suggested as a candidate for the virus that causes breast cancer. Therefore, it is reasonable to assume that some patients with breast cancer may have viral involvement and that TLR3 may be a receptor involved in immune response but also a carcinogenic process in breast. Involvement of nucleic acid in the pathogenesis of the disease and its implications in the modulation of immune response in therapy is an issue to be elucidated.

Keywords: TLR3. IFNγ. CXCR4. PKR. RNA. MMTV.

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LISTA DE SIGLAS E ABREVIATURAS

CCL5	Quimiocina (família CC) 5
CXCL10	Quimiocina (família CXC) 10
CXCL12	Quimiocina (família CXC) 12
CXCR4	Receptor de quimiocina (família CXC) 4
G-CSF	Fator estimulante de granulócitos
HERVS	Retrovírus endógeno humano
HMTV	Virus tumor mamário humano
HNSCC	Células escamosas de carcinoma de cabeça e pescoço
ICAM 1	Molécula de adesão inter celular 1
IL-6	Interleucina 6
IL-8	Interleucina 8
IL-12	Interleucina 12
INFα	Interferon do tipo alfa
INFβ	Interferon do tipo beta
IFNγ	Interferon do tipo gama
IRF	Fator Regulador de Interferon
IRF-3	Fator de Regulação do Interferon
LRR	Repetições em Leucina
MAP	Proteína Quinase Ativada por Mitógeno
MDA5	Gene 5 Associado a Diferenciação de Melanoma
MHC	Complexo de Histocompatibilidade Humano
MIP1	Proteína inflamatória de macrófago 1
MMTV	Vírus tumor mamário de camundongo
MyD88	Proteína Adaptadora
NF-κB	Fator Nuclear Kappa B
PAMPs	Padrões moleculares associados ao patógeno
PKR	Proteína Quinase Dependente de RNA
Poli (I:C)	Ácido Poliriboinosínico:Poliribocitidílico
RIG-I	Gene I Induzível de Ácido Retinóico
RNA	Ácido Ribonucléico
RNAds	RNAs Dupla Fita

TIR	Receptor Toll de Interleucina
TLR3	Receptor Toll like 3
TLRs	Receptores Toll like
VCAM1	Molécula de adesão celular vascular 1.

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1 INTRODUÇÃO

1.1 RECEPTOR TOLL LIKE

Contrariando as definições tradicionais que dividem o sistema imune em inato e adaptativo, atribuindo papéis específicos a cada um, há um crescente acúmulo de evidências atribuindo ao sistema inato um papel mais significativo na defesa no organismo humano.

Os TLRs compreendem uma família de receptores de proteínas de superficie celular e possuem importante função na resposta imune inata. Estes receptores estão presentes em diferentes tipos de células, que funcionam em mamíferos, reconhecendo componentes moleculares de microorganismos, os quais são denominados padrões moleculares associados ao patógeno (PAMPS), sendo um dos principais mecanismos pelo qual o hospedeiro reconhece que existe um microrganismo presente. Essa importante informação é transmitida através da membrana celular ao núcleo, onde genes especificos podem ser ativados para desencadear uma resposta apropriada. Esses receptores são homólogos a familia do receptor de interleucina 1 (IL-1), em humanos, e a proteína *Toll* da *Drosophila* (TRINCHIERI, 2007). TLRs são formados por domínio extracitoplasmático de repetições em leucina (LRRs) as quais estão envolvidas diretamente ou através de moléculas acessórias ligando ao domínio que interage com domínio receptor toll de interleucina 1 (TIR) contendo moléculas adaptadoras (BELL et al., 2003). Os TLRs ativam a via do fator nuclear kappa B (NF-κB) e fator regulador de interferon (IRF) que regulam a expressão de citocinas (TRINCHIERI, 2007).

No genoma humano, TLRs foram identificados: TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9 e TLR10 (AKASHI-TAKAMURA; MIYAKE, 2008), sendo que estes reconhecem e se ligam a PAMPs específicos: por exemplo, TLR4 e TLR5 são os receptores de lipopolissacarídeos e flagelina, respectivamente. Com base na localização celular, TLRs são classificados em dois grupos: TLRs de membrana plasmática incluindo TLR1, TLR2, TLR4, TLR5 e TLR6 e TLRs endossomal incluindo TLR3, TLR7, TLR8 e TLR9 presentes nos endossomas intracelular (MEDZHITOV, 2007).

Receptores *Toll-like* podem ser encontrados na membrana celular, no citosol e no endossoma. Células dendríticas, macrófagos, *natural killer*, e

mastócitos expressam receptores *Toll-like 3* (TLR3) intracelularmente e contribuem para a resposta imune inata (HEINZ et al., 2003; ORINSKA et al., 2005; WANG et al., 2006). Porém, em fibroblastos e células epiteliais TLR3 é expresso no endossoma e também na superfície das células (MATSUMOTO et al., 2002; 2003).

Estudos destinados a decifrar os caminhos da sinalização através de TLR3 em diferentes subconjuntos de células dendríticas podem fornecer informações importantes facilitando a utilização de RNAs dupla fita (RNAds -*double stranded*) como um ativador das células dendríticas (GAUZZI et al., 2010).

Os RNAds virais participam na apoptose de células infectadas por vírus, mas o caminho da sinalização não esta totalmente esclarecido. Salaun e colaboradores (2006) demonstraram que RNAds sintético induz apoptose em células de câncer de mama humano de uma maneira dependente de TLR3, o qual envolve o adaptador molecular T*oll*/IL-1R que induz IFN-beta (IFNβ) e sinalização autócrina, e independentemente da proteína quinase ativada por RNA (PKR). A atividade pró-apoptótica endógena humana de TLR3 expresso por células cancerígenas revelam um novo aspecto da biologia do TLR, o qual pode trazer um avanço clínico utilizando antagonistas de TLR3 como agente citotóxico em câncer.

1.2 RNA POLI (I:C) E TLR3

O ácido poliriboinosínico:poliribocitidílico (poli (I:C)) é um análogo sintético de RNA dupla fita, sendo um modelo molecular associado à infeção viral. O TLR3 reconhece estes RNAs e ativa genes que aumentam as citocinas inflamatórias e moléculas co-estimulatórias, importantes nas interações celulares (TAKEDA et al., 2003; MATSUMOTO; SEYA, 2008).

Alexopoulou e colaboradores (2001) demonstraram que o TLR3 humano reconhece RNAds, e a ativação deste receptor por poli (I:C) pode induzir a ativação de NF-kB e proteína quinase ativada por mitógeno (MAP) independentemente de MyD88 (proteína adaptadora), e causar a maturação de células dendríticas.

Sabe-se que RNAds, além de desencadear a sinalização pró-inflamatória em cascata que leva à produção de IFN, também ativa diretamente algumas enzimas, incluindo a PKR (DE LUCCA et al., 2002; WATANABE et al., 2003; 2005) e 2'- 5' oligoadenilato sintase (SAMUEL, 2001), e helicases mais

recentemente identificada como gene I induzível de ácido retinóico (RIG-I) e gene 5 associado a diferenciação de melanoma (MDA5) (UNTERHOLZNER et al., 2008).

RNAds ativa duas vias importantes, sendo a via NF-kB/MAP kinase, e a outra envolvendo o fator de regulação do IFN (IRF-3) e produção de IFNβ. O resultado é a ativação e aumento da regulação de genes importantes para a atração de células imunes (TRINCHIERI, 2007).

Okahira e colaboradores (2005) analisaram a estrutura do RNAds capaz de induzir a produção de IFNβ através do TLR3 utilizando RNA dupla fita sintético e RNAds viral, e verificaram que este reconhecimento é feito preferencialmente utilizando RNA sintético (poli (I:C)).

Estudos realizados por Liu e colaboradores (2008) verificaram que poli (I: C) induz o aumento da regulação do TLR3, eotaxina e CCL5 (RANTES) e, a expressão de IL-6, IL-8, G-CSF, MIP-1, ICAM-1 e VCAM-1. Portanto estas células podem desempenhar um papel importante na modulação da resposta imune local, na presença de poli (I:C).

A estimulação de TLR3 por poli (I: C) induz a apoptose em células de câncer de maneira dose-dependente. Estudos realizados por Nomi e colaboradores (2010) demonstraram a atividade pró-apoptótica de TLR3 expressa por células escamosas de carcinoma de cabeça e pescoço (HNSCCs) e esses resultados sugerem que TLR3 poderia ser um novo alvo para terapia em HNSCCs.

Pesquisa realizada por Tabiasco e colaboradores (2006) demonstrou que TLR3 também está presente nas células que participam diretamente da resposta imune adaptativa como linfócitos T $CD8^+$ humano. Neste contexto, TLR3 atua aumentando a produção de INFγ por estas células, evidenciando que TLR3 é um receptor com potencial na imunidade inata e adaptativa (VERCAMMEN et al., 2008; ZHANG et al., 2009).

Interferons (IFN) são proteínas com funções imunomodulatórias, citostáticas e com atividade citotóxica. Envolvidos tanto na resposta imune inata como na adaptativa, participam e modulam também a resposta imune do hospedeiro frente aos tumores (SELIGER et al., 2008). O INFγ é produzido em grande quantidade principalmente por células T (YOUNG; ORTALDO, 1987), e tem se mostrado um potente fator de sobrevivência das células B em culturas celulares obtidas de pacientes com leucemia linfocítica crônica tendo, portanto, efeitos

antiapoptóticos (BUSCHLE et al., 1994). O INFγ é o principal fator de ativação de macrófagos, com numerosas funções, incluindo atividade antimicrobiana (NATHAN et al., 1983), aumento do potencial microbicida contra patógenos intracelulares (TORRICO et al., 1991), estímulo da apresentação de antígenos aos linfócitos através da indução de moléculas MHC de classe II (BASHAM; MERIGAN, 1983) e promoção da citotoxicidade a tumores (PACE et al., 1983).

1.3 TLR3 E O ENVOLVIMENTO COM RNA ENDÓGENO NA IMUNOMODULAÇÃO

Tem sido relatado que RNA autólogo possui um papel imunomodulador na artrite reumatóide via TLR3. Brentano e colaboradores (2005) demonstraram que o RNA liberado de células necróticas pode atuar como um ligante endógeno para TLR3 promovendo estimulação da expressão de genes pró-inflamatórios de fibroblastos sinoviais na artrite reumatóide.

Karikó e colaboradores (2004) demonstraram que RNA heterólogo liberado por células necróticas também estimula TLR3 e induz a ativação do sistema imune. É possível que o RNA liberado a partir de células mortas, contenha estruturas de dupla fita que ativam TLR3.

Em outro estudo realizado por Karikó e colaboradores (2005) foi verificado que uma variedade de RNAs naturais possuem capacidades diferentes para ativar células imunes. Os RNAs mais eficazes neste experimento foram os que apresentaram o menor número de nucleotídeos modificados e, portanto, existe a hipótese de que a modificação dos nucleotídeos suprima o efeito imuno-estimulador de RNA.

O RNA, provavelmente, através da estrutura secundária, pode ser um modulador de TLR3. Este fato tem relevância fisiológica porque RNA liberado por tecidos danificados ou dentro das células endocitadas poderia servir como um ligante endógeno para TLR3 que induz ou modula a resposta imune.

Um membro pertencente aos transcritos não codificadores de mamíferos foi identificado como *noncoding transcript in T cell* (NTT) RNA, o qual foi reconhecido como gene diferencialmente expresso em células T $CD4^{+}$ humanas. Este gene apresenta um transcrito de 17kb seletivamente expresso em células T ativadas (LIU et al., 1997). NTT parece estar envolvido com a ativação de células (macrófagos) através de IFNγ. Células podem utilizar diferentes mecanismos de

regulação da síntese de RNA e neste contexto a expressão do gene NTT poderia modular a resposta dos linfócitos T (AMARANTE et al., 2005).

1.4 TLR3 e RNA: Implicações na Terapia Anti-tumoral

Sabe-se que TLRs são expressos não só em células do sistema imunológico, mas também em células tumorais, indicando que TLRs podem desempenhar importantes papéis na biologia tumoral (CONROY et al., 2008). É conhecido que a persistência do processo inflamatório pode induzir a formação de tumores. Esta ocorre, em parte, porque citocinas e quimiocinas, desempenham um papel crucial na promoção da angiogênese, metástases e ativação do sistema imune adaptativo (COSTANTINI et al., 2009).

Zhang e colaboradores (2009) analisaram o efeito da ativação TLR3 na metástase do carcinoma da nasofaringe. Verificou-se que a ativação de TLR3 diminui a expressão do receptor de quimiocina CXCR4 de uma maneira dose-dependente e inibe a migração celular em resposta ao ligante do CXCR4, o fator deridado de células estromais-1alfa (SDF-1α ou CXCL12), em ensaios de quimiotaxia. A ativação de TLR3 reduziu significativamente a capacidade das células de carcinoma da nasofaringe para formar metástases em linfonodos drenantes, quando injetado em camundongos atímicos.

Estudos realizados por González-Reyes (2010) verificaram que a expressão aumentada de TLR3 esta associada com alta probabilidade de metástases, o qual esta de acordo com outros estudos indicando que a expressão TLR3 está relacionada com a agressividade tumoral (SALAUN et al., 2006; SHOJAEI et al., 2009; SCARLETT et al., 2009; ALLHORN et al., 2008; MORIKAWA et al., 2007). Portanto, TLR3 pode representar um bom alvo terapêutico no câncer de mama.

A extensão e a qualidade das modificações dos nucleotídeos podem alterar a eficácia tanto para RNA endógeno quanto para RNAs patogênicos. Karikó e Weissman (2007) descreveram algumas modificações que ocorrem naturalmente no RNA e sua influência sobre a capacidade destes RNAs em ativar as células imunes e TLRs. RNAs contendo nucleotídeos modificados têm grande importância em aplicações clínicas. Vários estudos têm demonstrado que a ativação de TLR3 por RNAds inibe diretamente a proliferação celular e induz apoptose em células tumorais

(LE et al., 2008; SALAUN et al., 2006; 2007; TAURA et al., 2010). Neste contexto, a investigação da utilização de compostos derivados de RNAds em combinação com agentes anticâncer tem sido promissor.

RNAs potencialmente ativados *in vitro* ou recém isolados de células dendríticas humana ativadas, levou à produção e expressão aumentada de IL-12, IFNα e moléculas co-estimulatórias. Estes dados podem auxiliar o desenvolvimento de aplicações terapêuticas utilizando vacinas baseadas em RNA ou como um adjuvante na imunoterapia (KARIKÓ et al., 2005).

O análogo sintético poli (I: C) pode produzir efeitos secundários tóxicos *in vivo*, incluindo choque, insuficiência renal, coagulopatias e reações de hipersensibilidade (ROBINSON et al., 1976). A modificação de poli (I: C) através da introdução de bases não pareadas (uracila e guanina) resulta em RNAds capaz de sofrer hidrólise acelerada com baixa toxicidade em humanos. Poli (I:C 12U) é um RNAds sintético, conhecido como Ampligem®, avaliado como não tóxico e utilizado como um adjuvante na imunoterapia do câncer (Navabi et al., 2009). Verificou-se que monócitos e células dendríticas humanas derivadas de lisado tumoral e ativadas com Ampligen são capazes de gerar respostas Th1 específica contra o câncer (JASANI et al., 2009).

O envolvimento do RNA endógeno com receptor TLR3 e a liberação de várias citocinas e quimiocinas na patogênese de doenças imunológicas e outras doenças como câncer, ainda esta sendo investigada e pode ter implicações importantes no futuro. Conhecimentos adquiridos a partir destes estudos podem auxiliar na compreensão de várias doenças onde RNAs podem desempenhar um papel importante na patogênese ou na regulação da expressão gênica.

1.5 RNA de Origem Viral em Tecido Mamário Tumoral

Muitos fatores de risco estão envolvidos na etiologia do câncer de mama, como, idade, dieta, alterações hormonais, além de predisposição genética. Estudos sugerem que agentes virais podem estar relacionados com a doença. De acordo com Etkind e colaboradores (2008), dados moleculares e epidemiológicos indicam um possível envolvimento do *mouse mammary tumor virus* (MMTV), agente etiológico de neoplasia da glândula mamária em camundongos em laboratório, em uma porcentagem pequena de casos de câncer de mama em humanos.

O MMTV é retrovírus tipo B, descoberto em 1936, e pode causar câncer de mama em camundongos por um processo chamado mutagênese por inserção. Durante a replicação do MMTV, sua seqüência de DNA pode ser inserida próxima ou dentro de oncogenes, responsáveis pelo controle do crescimento e divisão celular. Geralmente, ocorre perda de função deste supressor de tumor e estas mutações serão responsáveis pela formação de tumor (CARDIFF et al., 1968; ETKIND et al., 2000).

O genoma humano apresenta seqüências endógenas denominadas HERVs (*human endogenous retroviruses*), que são componentes naturais, similares aos retrovírus exógenos, incluindo o MMTV (FRANK et al., 2008; INDIK et al., 2007). Tem sido demonstrado um vírus do tipo HERV em câncer de mama em humanos, o *human mouse mammary virus* (HMTV), que apresenta 95% de homologia com MMTV (MELANA et al., 2007; FRANK et al., 2008).

Indik e colaboradores (2007) sugeriram a capacidade de replicação viral do MMTV em células humanas. De acordo com Levine e colaboradores (2004), existem diferenças demográficas na prevalência de câncer de mama associado ao MMTV. Foi observado maior porcentagem de detecção de MMTV em pacientes com câncer de mama na Tunísia (74%) comparado com a prevalência nos Estados Unidos (36%), Itália (38%), Austrália (42%), Argentina (31%) e Vietnã (0,8%). Sequências do envelope do MMTV foi também identificado em câncer de mama humano na Australia, Argentina, China, Itália, Mexico, Tunisia, e Estados Unidos, e raramente encontrado em tecido mama saudável (LAWSON et al., 2010). Portanto, é razoável supor que algumas pacientes com câncer de mama podem apresentar envolvimento com MMTV, porém este é ainda um tema a ser elucidado.

2 OBJETIVOS

2.1 OBJETIVO GERAL

Avaliar as possíveis implicações do RNA humano endógeno na imunomodulação através de TLR3, bem como avaliar as implicações deste receptor no microambiente pró-inflamatório do câncer de mama.

2.2 OBJETIVOS ESPECÍFICOS

2.2.1 Cultura de Células Mononucleadas do Sangue Periférico

a Analisar a imunofenotipagem para CD4, CD8 e CD3 por citometria de fluxo das células PBMCs sensibilizadas ou não com poli (I:C) e RNA endógeno.

b Verificar a toxicidade dos diferentes estímulos (poli (I:C) e RNA), nas PBMCs, através da quantificação da desidrogenase láctica.

c Analisar a expressão gênica de TLR3, INFγ, CXCR4 e PKR nas culturas de células,

d Comparar a expressão gênica de TLR3, IFNγ, CXCR4 e PKR em cultura de células ativadas com poli (I:C) e RNA endógeno.

2.2.2 Células de Tecido Mamário de Pacientes com Câncer de Mama

a Avaliar a expressão gênica de TLR3 no tecido mamário saudável e tumoral;

b Comparar a expressão gênica de TLR3 e IFNγ, e TLR3 e CXCR4 em tecido mamário saudável e tumoral.

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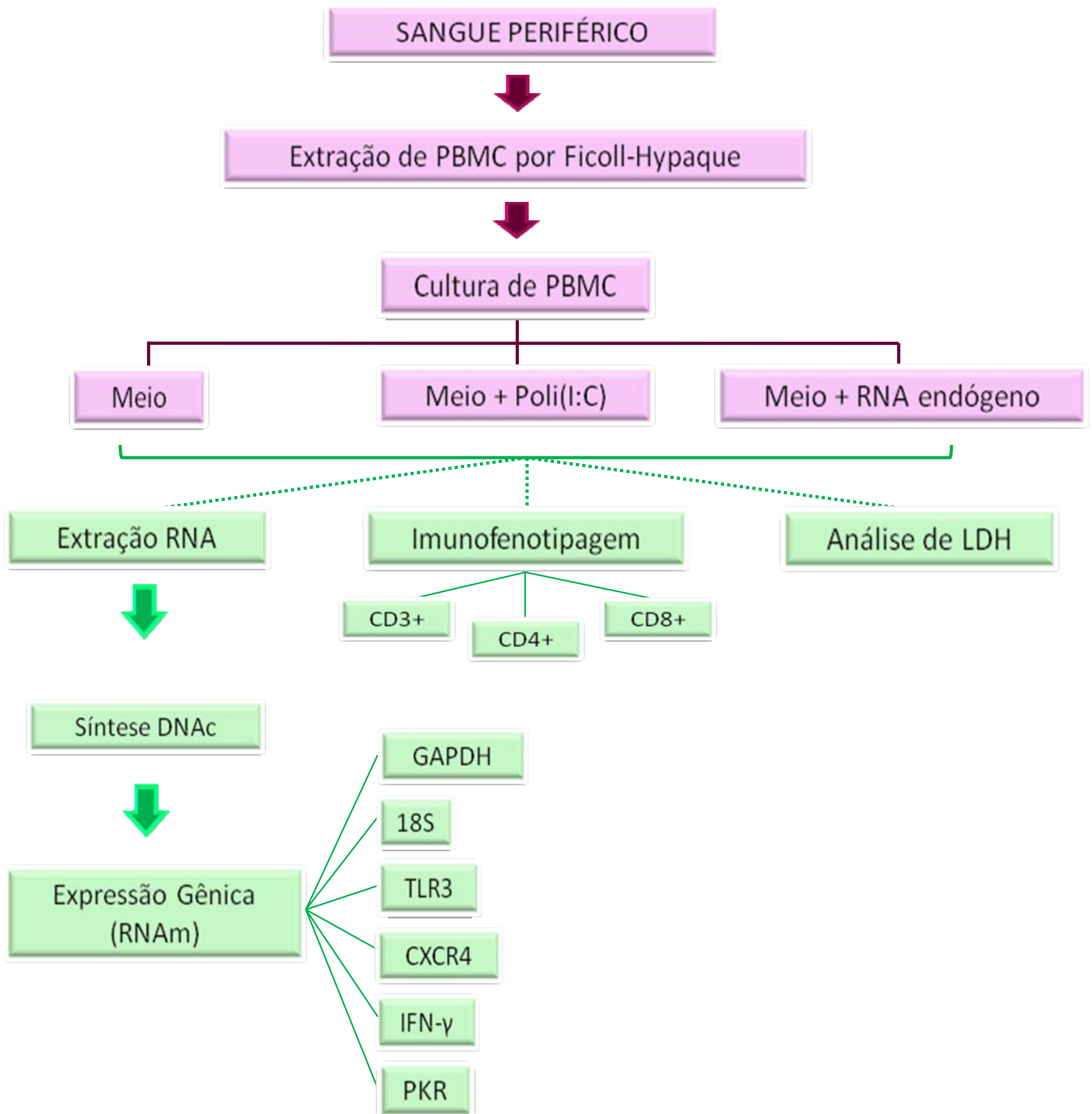
3 PRODUÇÃO CIENTÍFICA

3.1 RECEPTOR TOLL-LIKE 3: POSSÍVEL IMUNOMODULAÇÃO ATRAVÉS DE RNA

A investigação da imunologia envolvendo RNA tem sido amplamente explorada com o reconhecimento da existência do receptor Toll like 3 (TLR3) o qual interage com RNAs de origem viral, RNAs sintéticos (poli (I:C)) e RNA de origem endógena e sua estimulação resulta na liberação de citocinas e quimiocinas proinflamatórias. A extensão e a qualidade das modificações dos nucleotídeos podem alterar a eficácia tanto para RNA endógeno quanto para RNAs patogênicos. Estudos têm demonstrado que a ativação de TLR3 por RNAds inibe diretamente a proliferação celular. RNA proveniente de células necróticas do líquido sinovial de artrite reumatóide ativa fibroblastos sinoviais via TLR3 e o RNA liberado a partir de células necróticas pode atuar como um ligante endógeno para TLR3 promovendo estimulação da expressão de genes pró-inflamatórios de fibroblastos sinoviais na artrite reumatóide. A nossa proposta foi investigar se RNA proveniente de células do sangue periférico humano autólogo seria capaz de imunomodular a expressão de genes como TLR3, CXCR4, IFNγ e PKR em PBMCs.

Delineamento Experimental I



Artigo 1

Human Endogenous RNAs: Implications for Immunomodulation of Toll-like Receptor 3

Abstract: Nucleic acids can be recognized by TLRs among mammalian receptors in which TLR3 recognizes double-stranded (ds)RNA, a product from replication of some viruses. Polyinosinic-polycytidylic acid, referred to as poly (I:C), an analog of viral dsRNA, interacts with TLR3 and thereby elicits immunoinflammatory responses characteristic of viral infection or down regulated the expression of chemokine receptor CXCR4. It is known that dsRNA also directly activates IFN-induced enzymes, including the RNA-dependent protein kinase (PKR). The mRNA expression of TLR3, CXCR4, IFNγ and PKR was investigated in culture of peripheral blood mononuclear cells with poly (I:C) and endogenous RNA from human PBMC. There was no citotoxic effect on cells or proliferation of $CD3^+$, $CD4^+$ and $CD8^+$ cells. The levels of TLR3 expression in PBMC in the presence of the poly (I:C) group was up-regulated 9.5 fold and for PBMC treated with endogenous the RNA was down-regulated 1.8 fold (p=0.002). The same performance was verified for IFNγ where in the presence of poly (I:C) there was an 8.7-fold increase and in the presence of endogenous RNA a 3.1 reduction in IFNγ was observed. For culture activated with poly (I:C), mRNA increased to CXCR4 (8.0 fold) and to PKR (33.0 fold), and these genes decreased in culture with endogenous RNA when compared with culture without stimulus. Thus high expression of mRNA for TLR3, INFγ, CXCR4 and PKR was verified in the presence of poly (I:C) and low expression in culture cells with endogenous RNA. In conclusion, TLR3 may have major physiological roles that are not in the context of viral infection. It is possible that RNA released from cells, could contain enough double stranded structures to regulate the cell activation. The involvement of self endogenous RNA endogenous for gene expression, and its implications in the regulation, are still being studied, and will have important implications in the future.

Keywords: TLR3. CXCR4. INFγ. PKR. RNA.

Introduction

The innate immune system is the first line of defense against invading pathogens (1). This system uses TLRs to recognize conserved pathogen-associated molecular patterns and orchestrate the start of immune responses. Several TLRs recognize and respond to nucleic acids. Double-stranded (ds) RNA, a frequent viral constituent, has been shown to activate toll-like receptor 3 (TLR3) (2,3). Data has demonstrated that TLR3 regulates amplification events during inflammation mediated by nonviral mechanisms (4). It is known that dsRNA-activated dendritic cells induce increase in Th1 and decrease in Th2 differentiation, resulting in

extremely polarized responses relative to those induced by unstimulated and other TLR ligand-activated dendritic cells (5).

In recent years, diverse combinations of drugs targeting multiple pathways have been used for cancer treatment. One of the agents for tumor chemotherapy that has been assessed with favorable outcome in clinical trials is the synthetic dsRNA (6,7). The ability of dsRNA to directly stimulate TLR3 and produce type I interferons (IFNs) was primarily the rationale for its clinical use in cancer patients (8). More recently, several studies have demonstrated that the activation of TLR3 by dsRNA directly inhibits cell proliferation and induces apoptosis in tumor cells (9-11). In view of these promising effects, the use of dsRNA-derived compounds in combination with anticancer agents for chemo-immunotherapy warrants robust investigation (12).

TLR3 mediates anti-viral immune response by activating the innate immunity and cross-priming $CD8^+$ T cells. TLR3 agonists can directly trigger apoptosis in human cancer cells, and have been used as adjuvants to treat cancer patients with the aim of inducing an IFN-dependent immune response. Chemokines receptors such as CXCR4, have been implicated in organ-specific metastasis of various cancers, and the results from functional chemotaxis assays indicated that the treatment of nasopharyngeal carcinoma cells with poly I:C dose-dependently reduced CXCR4 expression (13).

The double-stranded RNA-activated PKR has a key role in the innate immune response to viral infection in higher eukaryotes. PKR contains an N- terminal dsRNA-binding domain and a C-terminal kinase domain. In the prevalent auto inhibition model for PKR activation, dsRNA binding induces a conformational change that leads to the release of the dsRNA-binding domain from the kinase, thus relieving the inhibition of the latent enzyme.

Further discovery and characterization of RNAs help to understand innate immunity and thereby provide the opportunity to make new and better RNA-based therapeutics (14). The dsRNA, an intermediate virus replication and a signature of infection and dsRNA are recognized in the cytoplasm via PKR, RIG-I, and MDA-5 (15). These trigger the release of inflammatory cytokines, that is, they activate innate immunity which shapes adaptive immune response (16, 17).

The purpose of the present study was analyze the expression of mRNA for TLR3, CXCR4, IFNγ and PKR in PBMC with poly (I:C) and human endogenous RNA.

Materials and Methods

The Human Ethics Committee of the State University of Londrina approved the present study and a voluntary written consent term was obtained from all of the patients enrolled in the present study. Peripheral blood mononuclear cells (PBMC) were collected from healthy blood donors with negative sorology for HIV, HBV and HCV.

Cell Cultures

Peripheral blood cells were directly obtained in EDTA (EthyleneDiamine Tetraacetic Acid) vacutainers. The PBMC were extracted by Ficoll-Hypaque (Sigma-Aldrich Co. Ltd, St. Louis, MO, USA). The PBMC cells were maintained in RPMI 1640 plus 10% (v/v) heat-inactivated fetal bovine serum (FCS, GIBCO, Grand Island, NY) in 24-well plates (Costar, Austria) at 1×10^6 cells/well. All of the media were supplemented with 2 mM-glutamine, 100 units/mL penicillin G and 100 units/mL streptomycinin. The cells were maintained at 37°C in a humidified incubator containing 5% CO_2 and presence or absence of stimuli for 24 hours. Poly (I:C) (Sigma-Aldrich Co. Ltd, St. Louis, MO, USA) was used at 50µg/mL concentrations. Endogenous total RNA was obtained from PBMC from self donors of this study, the same way it is described in RNA isolation. It was used at the concentrations of 500µg/mL in the culture.

Quantification of Lactate Dehydrogenase (LDH)

The citoplasmatic enzyme LDH was quantified in all samples for the cytotoxicity analysis. The Dimension® (DADE Behring, Newark, USA) clinical chemistry system was used to determine the LDH activity. The lactic dehydrogenase method is a modification of the enzymatic lactate to pyruvate procedure modified by Gay et al. (18).

Flow cytometry analysis

A total of 1–3x 10^6 cells were saturated with purified normal mouse Ig (Becton Dickinson) at room temperature for 10 min. The cells were then incubated for 30 min at 4ºC with mouse monoclonal antibodies anti-CD3 labeled with fluorescein isothiocyanate (FITC), anti-CD8 labeled with phycoerythrin (PE) and anti-CD4 labeled with allophycocyanin (APC). The cells were counted by flow cytometry performed in the FACSCalibur™ flow cytometer (Becton Dickinson Biosciences, San Jose, CA, USA) equipped with 635 nm and 488 nm lasers that were capable of detecting light scatter (forward and side) and four-color fluorescence with emission detectable in four ranges: 515-545 nm, 562-607 nm, > 650nm and 652-668 nm.

RNA isolation and reverse transcriptase reaction

Total cellular RNA was extracted from culture cells with TRIzol LS reagent (Invitrogen™, Carlsbad, California, USA) according to the manufacturer's instructions. Purified total RNA was measured and assessed for purity by determining absorbance at 260 and 280 nm and was then stored at –80°C until testing. Reverse transcriptase reaction was performed using 500ng RNA, 20 units cloned Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT; Invitrogen™), 4 units Recombinant Ribonuclease Inhibitor (RNaseOUT™; Invitrogen™) under the following conditions: 2.5 µM oligo dT, 50mM Tris HCl pH 8.3, 75mM KCl, 1.5 mM $MgCl_2$, 1.25 mM dNTP, at 42°C for 60 min in a Hybaid PCR Sprint Thermal Cycler (Biosystems, Guelph, Ontario, Canada).

Molecular analysis of Beta-actin mRNA

PCR for beta-actin was determined as described by Amarante et al. (19). Briefly, cDNA synthesis was carried out as previously described and the PCR conditions were: 94ºC for 1 min followed by 35 cycles of 94ºC for 30 sec, 55ºC for 30 sec, 72º C for 1 min and finally, 72ºC for 10 min in a Biocycler (Biosystems, Guelph, Ontario, Canada). PCR products were analyzed by electrophoresis on acrylamide gel (10%) and detected by a nonradioisotopic technique using a commercially available silver staining method.

Quantitative real-time PCR Conditions

Real-time PCR using SYBR green fluorescence was performed with 80 ng cDNA. Each real-time PCR reaction consisted of 2.5 uL RT product, 10uL Platinum®SYBR Green qPCR SuperMix UDG (Invitrogen™) and 0.25 uM of each sense and antisense primer. The amount of cDNA was estimated by the quantitative polymerase chain reaction (qPCR) amplified using the sense and the antisense primer according to table below (table 1). All the PCR reaction was performed for 40 cycles and the condition of each cycle was as follows: 95°C for 30 sec, annealing temperature for 30 sec and 72°C for 30 sec using a Chromo4™ Real Time PCR Detection (Bio-Rad, Hercules, USA). The Ct values (the cycle number at which emitted fluorescence exceeded an automatically determined threshold) reported are the mean fold change + SEM for three independent determinations. Data from the control PBMC and activated PBMC with poly (I:C) or RNA, show mean of Ct values, which are adjusted Ct values for CXCR4, TLR3 and INFγ that are corrected by Ct values for GAPDH and PKR that is corrected by Ct values for 18S from control samples, considering efficiency values, according to the Pfaffl method (20). Subsequently, a melting curve was recorded between 50 and 98°C with a hold every 2 sec.

Table 1 – Primers used in reactions of qPCR.

Gene mRNA	GenBank Acession Number	Primers	Sequence 5'- 3'	Annealing Temperature (TºC)
GAPDH	NM_002046	Forward reverse	GAA GGT GAA GGT CGG A GGG TCA TTG ATG GCA AC	54.0
TLR3	NM_003265	Forward reverse	AAA TAG ACA GAC AGA CAG AACAGT AAA AAC ACC CGC CTC AAA	54.0
CXCR4	AF025375	Forward reverse	TCTACTCCATCATCTTCTTTA ACGTTGGCAAAGATGAAGGTC	54.0
IFNγ	NM_000619	Forward reverse	AAT TGT CTC CTT TTA CTT CA GTCATC TCG TTT CTT TTT GT	54.0
PKR	M85294	forward reverse	ACA GCA AAA ATA GTT CAA GGT CA AAA GAG TTC CAA AGC CAA AA	57.0
18S	NR_003286	forward reverse	GTA ACC CGT TGA ACC CCA TT' CCA TCC AAT CGG TAG TAG CG	57.0

Statistical Analysis

Statistical analysis was carried out using the SPSS Statistics 17.0 program (SPSS inc., Chicago, Illinois, USA). A *p* value ≤0.05 indicated statistical significance. The paired samples correlations for relative expression were tested using t tests.

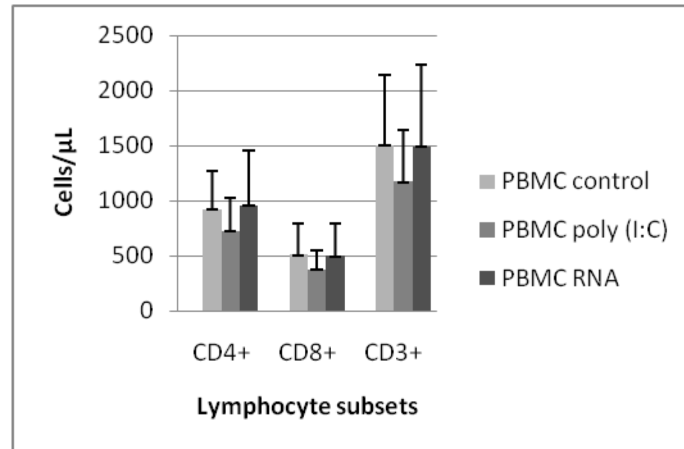
Results

Culture was performed with PBMC from 6 healthy blood donors in the presence and absence of double-stranded RNA poly (I:C) and endogenous RNA. Poly (I:C) was used for cell activation at a concentration of 50 µg/mL. Endogenous RNA was obtained from the same PBMC-blood donors and each PBMC culture was sensitized with the self endogenous RNA.

In the first step, after 24 hr, PBMC toxicity was analyzed by quantifying the lactate dehydrogenase (LDH). The concentration of LDH found in cultures was PBMC control 70.33 (±17.39) U/L, PBMCs activated with poly (I:C) 47.0 (±12.29) U/L and PBMC with RNA 60.00 (±5.29) U/L. Statistical significance was not observed between the PBMC control and PBMC with poly (I:C) and PBMC RNA ($p>0.05$), and no toxicity was detected in any culture.

The next step was to determine the number of CD4, CD8 and CD3 antibody binding sites (ABS) on unstimulated and stimulated culture cells. The difference between culture cells and culture stimulated with poly (I:C) and endogenous RNA was not statistically significant (figure 1).

Figure 3 – Quantification of $CD4^+$, $CD8^+$ and $CD3^+$ antibody binding sites by flow cytometry. The $CD4^+$ PBMC control (918,25/µL (±352,19), PBMC poly (I:C) 723,00/µL (±304,63), PBMC RNA 958,75/µL (±492,89). To $CD8^+$ PBMC control 507,87/µL (±283,46), PBMC poly 372,33/µL (±174,72), PBMC RNA 494,75/µL (±299,95) and to $CD3^+$ PBMC control 1501,87/µL (±644,04), PBMC poly 1169,67/µL (±473,65), PBMC RNA1486,50/µL (±747,30).

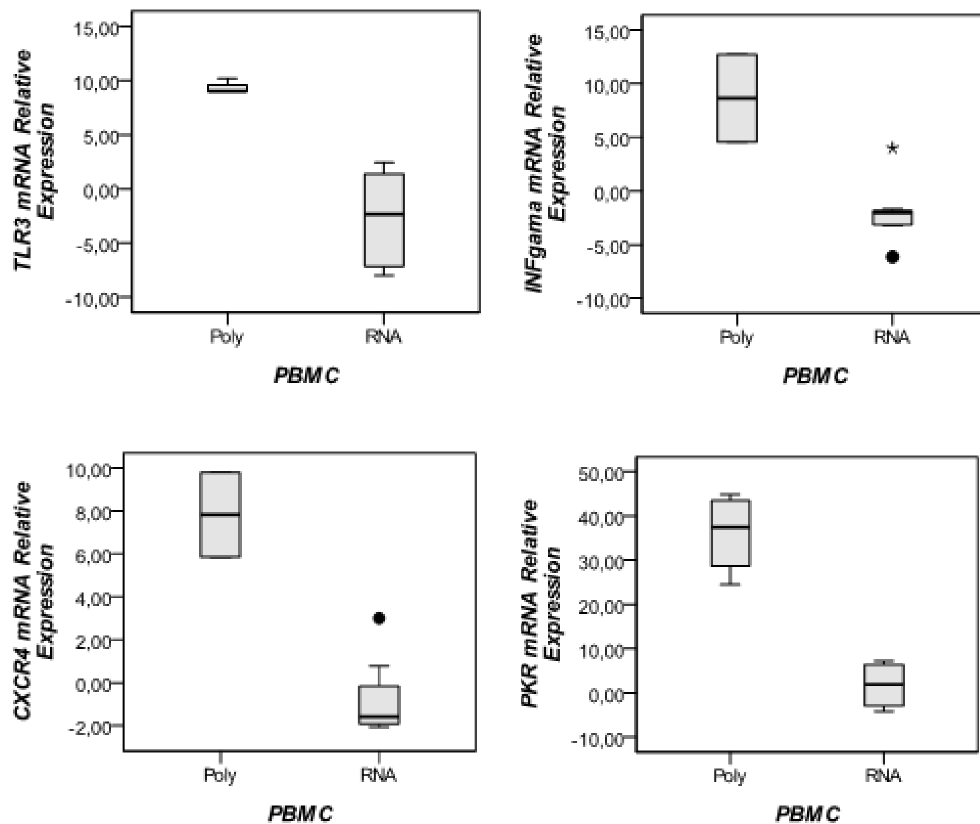


RNA was extracted from culture in the presence or absence of poly (I:C) and endogenous RNA for 24 hr at 37ºC and 5% CO_2. The viability and integrity of the RNA samples and cDNA quality were analyzed by conventional PCR for beta-actin, performed with specific primers. All the RNA samples presented detectable quantities of beta-actin mRNA and acceptable integrity during amplification. No contamination with genomic DNA was verified, since all the amplified products presented a fragment correspondent to 353 bp.

Quantitative PCR was used to investigate the expression of mRNA for TLR3, IFNγ, CXCR4 and PKR in human blood cells activated with poly (I:C) and endogenous self RNA. The levels of TLR3 expression in PBMC in the presence of the poly (I:C) group was up-regulated 9.5 fold and for PBMC treated with endogenous the RNA was down regulated 1.8 fold ($p=0.002$). The same performance was verified for IFNγ where in the presence of poly (I:C) there was an 8.7-fold increase and in the presence of endogenous RNA a 3.1 reduction in IFNγ was observed. For culture activated with poly (I:C), mRNA increased to CXCR4 (8.0 fold) and to PKR (33.0 fold), and these genes decreased in culture with endogenous RNA when compared with culture without stimulus (1,2 fold and 2,01 fold, respectively). This correlational was statistically significant ($p=0.001$). Thus high expression of mRNA for TLR3, INFγ, CXCR4 and PKR was verified in the presence of poly (I:C)

and low expression in culture cells with endogenous RNA, as shown in figure 2.

Figure 4 – The mRNA relative expression of TLR3, IFNγ, CXCR4 and PKR in culture cells in the presence of poly (I:C) and endogenous RNA. Pfafll values were compared between values from culture in the presence of poly (I:C) and culture with endogenous RNA.



Discussion

Pathogen recognition is largely assigned to an evolutionarily conserved family of receptors, the Toll-like receptors, which function in innate immunity and subsequent acquired immunity against microbial infection or tissue injury.

In the present study, CD4 and CD8 levels were uniform among lymphocyte subsets of $CD3^+$ cells and the culture with poly (I:C) and with endogenous RNA they did not present toxicity and lytic activity for 24 hours, which was demonstrated by LDH assay . All RNA used in this study was tested to integrity through beta-actin expression evaluation.

It has been described that the RNA released from necrotic synovial fluid cells activates rheumatoid arthritis synovial fibroblasts via TLR-3. This study from Brentano et al. (21) indicated that the RNA released from necrotic cells might act as an endogenous TLR3 ligand for the stimulation of proinflammatory gene expression in rheumatoid arthritis synovial fibroblasts. In our study we verified that endogenous RNA obtained from self healthy PBMC, in contrast to poly (I:C), reduced expression of TLR3, IFNγ, CXCR4 and PKR in PBMC when compared with PBMC alone, which was statistically significant.

Ben-Asouli et al. (22) has shown that human IFNγ mRNA activates the PKR kinase. IFNγ mRNA activates PKR through a pseudoknot in its 5'-UTR. This 5'-UTR creates a stem-loop with a remote sequence in the RNA molecule. Thus, IFNγ mRNA regulates its own translation by an RNA pseudoknot. Several reports have described messenger RNA-like transcripts as polyadenylated but with no defined open reading frames, indicating that they lack protein coding capacity and, therefore, regulatory RNAs exert their action at the RNA level (23,24).

Research by Tabiasco et al. (25) demonstrated that TLR3 is also present in cells that participate directly in the adaptive immune response in which human effector $CD8^+$ T lymphocytes. In this context, TLR3 ligation was shown to directly increase IFNγ production by $CD8^+$ T cells. This evidence indicated that TLR3 is a "danger" receptor with a pleiotropic potential in innate and adaptive immunity (26). These authors demonstrated that TLR3 contributes to the elimination of specific viruses, but others have demonstrated that some viruses can benefit from TLR3 stimulation. The general outcome is probably dependent on several factors, such as the type of virus, the viral load, its mode of infection (endoplasmic versus cytoplasmic), the cell type that is infected, and the stage of infection.

Primary attention has so far been focused on the role of TLR3 in eliciting cellular responses to virus infection, because of the antimicrobial functions of other TLRs and the fact that dsRNA, the ligand of TLR3, is produced during the replicative cycle of some viruses. The physiological roles of TLR3 remain to be clearly defined. It is possible that TLR3 has major physiological roles that are not in the context of viral infection. In that case, the origin of the dsRNA is unclear. It is possible that extracellular RNA released from cells, due to their apoptotic or necrotic death, contains enough double stranded structures to activate or inhibit the receptor.

In vitro transcribed mRNA or endogenous RNA released from necrotic cells, when added extracellularly, has been shown to activate experimental or natural cell expressing TLR3 (27).

PKR plays an important role in mediating the antiviral effects of IFNs, but in uninfected cells PKR is also implicated in regulating cell proliferation under normal conditions (28). Several studies have demonstrated that the activation of TLR3 by dsRNA directly inhibits cell proliferation and induces apoptosis in tumor cells (9-11, 29).

Karikó et al. (30) reported that a variety of natural RNAs had different capacities to activate immune cells. The most potent RNAs were those that had the least number of modified nucleosides, therefore, it was hypothesized that nucleoside modification suppresses the immune-stimulatory effect of RNA. In a quest to prove this, several novel lines of evidence were discovered of RNA- mediated immune activation.

Cell may employ different mechanisms to regulate RNA synthesis, in this context, it is noteworthy the expression of the noncoding T cell RNA could be implicated in the T lymphocyte response (19). In the context of adaptative immunity response, it was demonstrated that TLR3 is also present in cells that participate directly in the adaptive immune response in which the human effector T lymphocytes express TLR3 as a functional coreceptor. If there is an endogenous noncoding short or long human RNA, these molecules could be a candidate for TLR3 or other intracellular receptors.

TLR3 has gained recognition as a novel molecular target for cancer therapy because TLR3 activation by its synthetic ligand poly (I:C) directly causes tumor cell death (12). TLR3 agonists can directly trigger apoptosis in human cancer cells and have been used as adjuvants to treat cancer patients with the aim of inducing an IFN-dependent immune response. Zhang et al. (13) verified that TLR3 activation down regulated the expression of the chemokine receptor CXCR4 in a dose-dependent manner and inhibited cell migration in response to the CXCR4 ligand. In this work although poly (I:C) increased expression of CXCR4 on PBMC, the contrary effect was verified with endogenous RNA. Although poly (I:C) was found to be the potent IFNγ inducer, in this work, it is possible that endogenous RNA promotes regulation of the expression of CXCR4, IFNγ and PKR, and there are no involvement with TLR3.

The involvement of RNA and its receptors and the release of several cytokines and chemokines in the pathogenesis of immune disorders and other diseases, such as cancer, are still being studied and will have important implications in the future. Insights gained from study with endogenous RNA as noncoding regulatory RNAs could advance our understanding of various diseases where nucleic acids play a prominent role in the pathogenesis or regulation of gene expression, determine a role for nucleoside modifications on RNA, and give future directions for the design of therapeutic RNAs (31).

The investigation of RNA-based immunology has been reinvigorated with the observation that TLR3s interact with RNA. mRNA therefore joins the list of endogenous ligands for TLRs and is the first endogenous ligand described for TLR3. The presence of these host ligands during inflammation activates the immune system. The further finding that nucleoside modification alters RNA-mediated TLR signaling presents a mechanism for the long-observed differences in immunogenicity between bacterial, viral and mammalian RNAs. This is one of the first studies to comprehensively compare not only the effects of TLR ligands such as double stranded RNA on human PBMC activation, but more importantly, the inhibitory effect of self endogenous RNA on PBMC. The involvement of RNA modification in the pathogenesis of immune disorders and other diseases, and its implications in the therapeutics, are still being studied, and should be important implications in the future.

Conflict of Interest Statement

The authors declare to comply with all requirements for publication; there is no conflict of interest.

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Artigo 2

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Toll-Like Receptor 3: Involvement with Exogenous and Endogenous RNA

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The recognition of pathogens is assigned to an evolutionarily conserved family of receptors, the Toll-like receptors (TLRs). The investigation of RNA-based immunology has been reinvigorated with the observation that TLR3s interact with RNA (dsRNA of viral origin, poly (I:C) and endogenous RNA). Many RNAs, therefore, join the list of endogenous ligands for TLRs. The further finding that nucleoside modification alters RNA-mediated TLR signaling presents a mechanism for the long-observed differences in immunogenicity. The involvement of RNA modification in the pathogenesis of diseases, and its implications in the therapeutics, are still being studied, and will have important implications in the future.

Keywords TLR3, RNA, Poly (I:C), immune cells

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INTRODUCTION

Toll like receptors (TLRs) are pattern recognition receptors for ligand molecules derived from microbes or host cells, and TLR-ligand binding plays a key role in innate immunity and subsequent acquired immunity against microbial infection or tissue injury [1, 2]. This system can recognize conserved pathogen-associated molecular patterns (PAMPs) through TLRs expressed on immune cells but also on a number of non-immune cells [3–6]. Mammalian TLRs respond not only to pathogen-associated structures but also to host-derived molecules that are released from injured tissues and cells [7].

TLRs are located either on the cell surface or in intracellular vesicular compartments. The cell-surface TLRs, including TLR1, TLR2, TLR4, and TLR6, recognize microbial membrane lipids [8], whereas

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RIGHTSLINK

TLR3, TLR7, TLR8, and TLR9 recognize microbial nucleic acids in endolysosomes and initiate innate and adaptive immune responses [9–12]. Indeed, TLR9 is able to respond to mammalian DNA if expressed on the cell surface [12] and unmethylated DNA. TLR7 also responds to host-derived, single-strand RNA [13]. TLR7/9 in dendritic cells (DCs) also responds to self-derived RNA/DNA, respectively, and drive autoantibody production [11].

Nucleic acid sensing in endolysosomes rather than on the cell surface is thought to be a safety mechanism that prevents response to self–nucleic acid, because self–nucleic acids are rapidly degraded before reaching endolysosomes [12]. Viral nucleic acid is, on the other hand, protected by capsid proteins and is able to reach endolysosomes [11].

TLR3 mRNA has been detected in a number of human tissues including the placenta, pancreas, lung, liver, heart, lymph nodes, and brain [14–16], and TLR3 transcripts have been found in a variety of human and mouse immune cells, including T lymphocytes, natural killer (NK) cells, macrophages, mast cells, and $\gamma\delta$ T cells [17–22]. Human fibroblasts and epithelial cells express TLR3 both intracellulary and on the cell surface while monocyte-derived immature dendritic cells and myeloid dendritic cells only express TLR3 intracelllularly [23, 24].

There are notable differences between mouse and man in this respect; for example, human TLR3 is highly expressed in immature dendritic cells whereas mouse TLR3 is highly expressed in macrophages [25]. TLR3 agonists are also well established and potent activators of dendritic cell maturation [26]. Both activities probably contribute to the important role of TLR3 in antiviral immunity and adaptive immunity induction.

TLR3 recognize dsRNA and activate genes that increase inflammatory cytokines and co-stimulatory molecules important for immune cell interactions [27]. It is known that both natural and synthetic double-stranded (ds) RNA elicit interferon (INF) production [28, 29]. Several studies have suggested that human cells recognize particular spatial and steric organizations of dsRNA via putative cell membrane receptors and produce type I IFN [30–32]. Among the synthetic dsRNAs, polyinosinic-polycytidylic acid referred to as poly (I:C), a synthetic analog of dsRNA and a molecular pattern associated with viral infection, interacts with TLR3 and thereby elicits immunoinflammatory responses [33]. Poly (I:C) was found to be the most potent IFN inducer [30].

In addition to TLR3, dsRNA was recognized by several other cytosolic sensors, such as PKR, oligoadenylate synthases, and the more recently identified RNA helicases such as the retinoic acid inducible gene I (RIG-I) and melanoma differentiation-associated gene 5 (MDA5)

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[34, 35]. TLR3 and the RIG-I/MDA5 RNA helicases differ in their cellular location and ligand specificities, and induce antiviral responses via different signaling pathways [36, 37].

Poly I:C induces innate immune responses by two pathways [34]. In one pathway, poly I:C is internalized through endocytosis and recognized by TLR-3 [38–40]. Upon recognition, TLR-3 transmits signals via the adaptor protein Toll-IL-1 receptor (TIR) domain containing the adaptor molecule-1 (TICAM-1] (also called TIR domain-containing adapter inducing IFN-α (TRIF)). This activates the transcription factors interferon regulatory factor 3 (IRF-3), NF-kB, and AP-1, a complex of activating transcription factor 2 (ATF2) and JUN, leading to the induction of type I IFN (especially IFN-α), cytokine/chemokine production and dendritic cell maturation [41–45] (Figure 1). Although it is clear that TLR3 recognizes extracellular dsRNA and induces TICAM-1-mediated innate and adaptive immunity, the in vivo role in anti-viral responses is still controversial. In the second pathway (intracellular pathway), *transfected complexed* poly (I:C) (complexes between poly (I:C) and transfection reagents to facilitate cellular internalization) is recognized by the intracellular cytoplasmic sensors melanoma differentiation-associated gene 5 (MDA-5) and retinoic acid inducible gene I (RIG-I) which results in the activation of the transcription factors interferon regulatory factor 3 (IRF-3), NF-kB, and activating protein 1 (AP-1) [39, 40, 43]. Djafarzadeh et al. [43] suggested that the NF-kB signalling pathway might not be directly involved in poly (I:C) induced mitochondrial dysfunction in cultured human hepatocytes.

Production of IFNs by TLR3 signaling is independent of the adaptor protein, the myeloid differentiation factor 88 (MyD88) but IFN-α induction by TLR7, TLR8, and TLR9 requires MyD88 [27–33, 46–50].

Damage-associated molecular patterns derived from injured normal epithelial cells and necrotic cancer cells appear to be present at significant levels in the tumor microenvironment, and their stimulation of specific TLRs can foster chronic inflammation [49].

TLR3 AND IMMUNOLOGICAL NETWORK

Many TLR3 effects rely on cells of the innate immune system that either express TLR3 or respond to inflammatory mediators that are produced upon TLR3 signaling. Immune cells that express TLR3 and contribute to an innate immune response are dendritic cells, macrophages, natural killer cells, and mast cells [20, 41, 51].

In light of the growing application of dendritic cell-based immunotherapy approaches and for a deeper understanding of the dsRNA-induced immune responses, clarification of these issues is of

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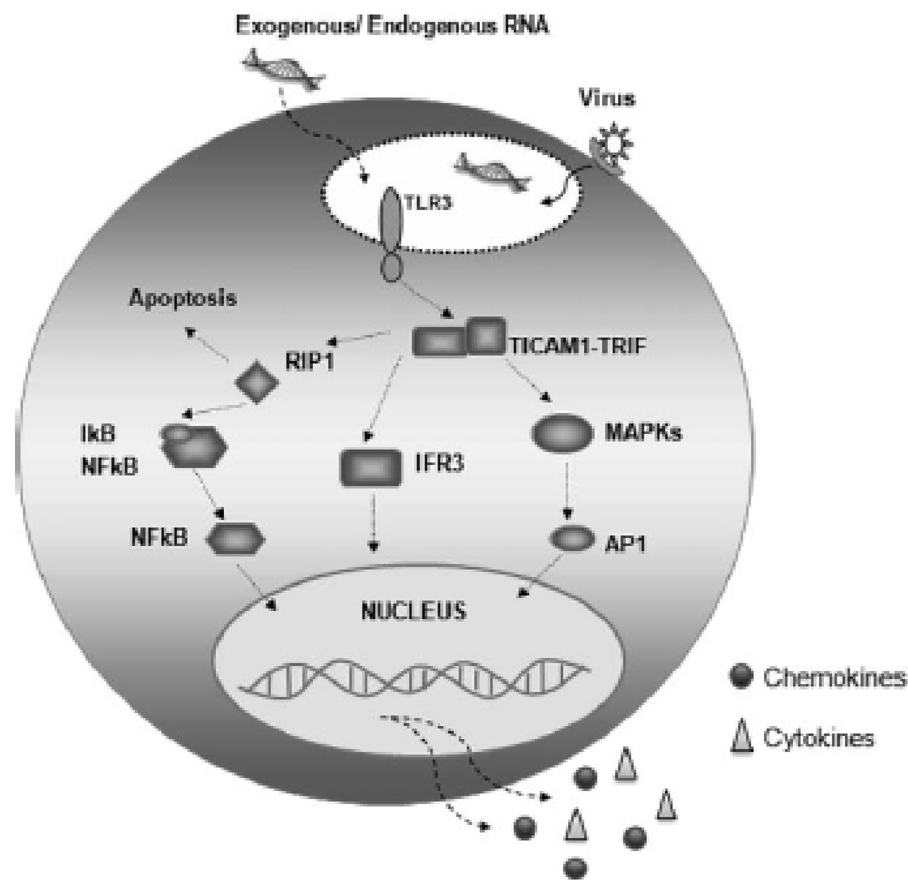

FIGURE 1 Toll-like receptor 3 ligands and signaling pathways. TLR3 are expressed in the endosomal membranes of various cells and recognize exogenous and endogenous ligands. TLR3 recognize dsRNA and activate genes that increase inflammatory cytokines and co-stimulatory molecules important for immune cell interactions. TLR3 recruit TICAM-1/TRIF and induce apoptosis by RIP1, and they also induce inflammatory cytokine/chemokine production by activation of interferon regulatory factor (IRF)3 NFkB and MAPK.

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great importance. Studies aimed at deciphering the TLR3 signaling pathways in different subsets of dendritic cells promise to provide important insights facilitating the use of dsRNA as an activator of dendritic cells and in vivo adjuvant [52].

The involvement of TLR3-TICAM-1 in activation of natural killer cells and citotoxic T lymphocytes by myeloid dendritic cells suggests that TLR3 serves as an inducer of cellular immunity sensing viral infection rather than a simple IFN inducer [26].

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Cross-talk between NK cells and DCs is critical for the potent therapeutic response to dsRNA, but the receptors involved remain controversial. Perrot et al. [53] also showed that polyinosinic-polycytidylic acid triggered human TLR3, RIG-I, and MDA5. This dsRNA enhanced NK cell activation within peripheral blood mononuclear cells (PBMCs) and induced IFN-γ. Poly (I:C) synergized with mDC-derived IL-12 for IFN-γ production by acting directly on NK cells. Finally, the requirement of both TLR3 and the Rig-like receptor (RLR) on mDCs and RLRs but not TLR3 on NK cells for IFN-γ production was demonstrated using TLR3- and Cardif-deficient mice and human RIG-I-specific activator. This reports the requirement of cotriggering TLR3 and RLR on mDCs and RLRs on NK cells for a pathogen product to induce potent innate cell activation.

Takahashi et al. [54] demonstrated that nasal mucosa-derived fibroblasts express a large amount of TLR3 mRNA. Stimulation with poly (I:C) directly induced production of IL-8 and RANTES in nasal fibroblasts. Viral respiratory infections are increasingly implicated in allergic exacerbations. Virus-induced activation of eosinophils through toll-like receptors (TLRs) could be involved. A study by Mansson et al. [55] was designed to examine TLR3 expression in eosinophils from bone marrow (BM) and peripheral blood (PB) during symptomatic allergic rhinitis. TLR3 expression was higher in BM-derived than in circulating cells and it was downregulated in both compartments during symptomatic allergic rhinitis. Stimulation with poly (I:C) increased the percentage of CD11b+ cells and enhanced the secretion of IL-8. Eosinophils activated via TLR3 might be more able to home and recruit leukocytes to inflammation sites.

Mast cells are long-lived CD34+-derived cells that migrate to inflammation sites and regulate innate immune responses via the production of cytokines, leukotrienes, and other inflammatory mediators [56]. Kulka et al. [57] demonstrated that mast cells produced type I IFNs after exposure to poly (I:C), respiratory syncytial virus, influenza virus, and type 1 reovirus, and expressed TLR-3 as well as all other known TLRs except TLR-10. In addition, TLR-3 signaling with poly (I:C) was uniquely capable of inducing the production of type I IFNs and did not result in the production of other proinflammatory cytokines.

TLR3 has also been implicated in the immunobiology of skeletal muscle. TLR3 is expressed intracellularly in muscle cells both in vitro and in vivo and is upregulated by dsRNA and IFN. TLR3 levels are high in muscle biopsy specimens from patients with inflammatory and human immunodeficiency virus-associated myopathies, suggesting a deleterious role for TLR3 in inflammatory muscle disease [58].

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The effects of poly (I:C) on the expression of proinflammatory cytokines, chemokines, and adhesion molecules; the signaling pathways that underlie such effects, were investigated in cultured human corneal fibroblasts. Liu et al. [59] verified that poly (I:C) induced the upregulation of TLR3, release of IL-6, IL-8, G-CSF, MIP-1β, eotaxin, and RANTES, and ICAM-1 and VCAM-1 expression in corneal fibroblasts. Therefore, corneal fibroblasts may play an important role in the modulation of local immune and inflammatory responses to viral infection in the corneal stroma.

Bérubé et al. [60] found that the nuclear factor kappaB (NF-kB) pathway is essential for the transcriptional regulation of both IL-8 and RANTES following the activation of TLR1/TLR2 and TLR3.

In the context of adaptative immunity response, research by Tabiasco et al. [21] demonstrated that TLR3 is also present in cells that participate directly in the adaptive immune response in which the human effector $CD8^+$ T lymphocytes express TLR3 as a functional coreceptor. In this context, TLR 3 ligation was shown to directly increase IFN-γ production by antigen-primed CD8+ T cells. This evidence indicates that TLR3 is a "danger" receptor with a pleiotropic potential in innate and adaptive immunity [61]. These authors demonstrated that many reports show that TLR3 contributes to the elimination of specific viruses, but others demonstrate that some viruses can benefit from TLR3 stimulation. The general outcome is probably dependent on several factors, such as the type of virus, the viral load, its mode of infection (endoplasmic versus cytoplasmic), the cell type that is infected, and the stage of infection.

Holm et al. [62] discovered that poly (I:C) induced synthesis of both IL-17A and IL-21 and that poly (I:C) was able to drive the differentiation of naive Th cells into an IL-21 but not into an IL-17A-producing phenotype. Finally, they found that the IL-21-producing cells that were differentiated in response to poly (I:C) expressed the chemokine receptor CXCR3, which is important in the recruitment of T cells into inflamed joints in rheumatoid arthritis. This is the first report to show that the TLR3 ligand poly (I:C) can directly induce IL-17A and IL-21 synthesis and drive differentiation of human naive CD4+ T cells.

Interestingly, TLR3 stimulation by poly (I:C) induced apoptosis in cancer cells in a dose-dependent manner. The research of Nomi et al. [63] demonstrated that TLR signaling affects survivin-mediated signal transduction in apoptosis. These results suggest that TLR3 could be a new target for therapy for human head and neck squamous cell carcinomas.

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ENDOGENOUS RNA AS A TLR3 LIGAND

It is known that poly (I:C) in a cell-associated form has been reported to be more efficient in triggering TLR3 than soluble dsRNA, suggesting that dsRNA from dying cells is probably a more potent and physiologically relevant TLR3 ligand than dsRNA from live cells [64].

Karikó et al. [65] demonstrated that a variety of natural RNAs had different capacities to activate immune cells. The most potent RNAs were those that had the least number of modified nucleosides; therefore, it was hypothesized that nucleoside modification suppresses the immune-stimulatory effect of RNA. In a quest to prove this, several novel lines of evidence were discovered about RNA-mediated immune activation.

Brentano et al. [66] demonstrated that mRNA containing short dsRNA sequences, which are released from necrotic synovial fluid cells, are sufficient to activate cultured human RASFs (synovial fibroblasts), resulting in the induction of expression of Th1-associated chemokines, such as CXCL10 and CCL5.These results suggest an important role of TLR-3 in the activation of synovial fibroblasts in rheumatoid arthritis. These findings extend those of a study that reported the activation of human endometrial cells with U1 RNA, has a significant double-stranded secondary structure—the U1 small nuclear RNP (U1 snRNP), capable of inducing innate immune responses consistent with TLR-3 ligation. Translocation of the U1 RNA–containing U1 snRNP out of the nucleus to locations where it could potentially encounter TLRs occurs in dead and dying cells [67, 68]. Thus, failure to clear dead cell debris may provide not only autoantigen targets for autoimmune responses but also a potentially proinflammatory signal via RNA interaction with TLR-3 [69].

Several studies have shown the presence of liver mitochondrial dysfunction during sepsis, and TLR3 ligand amplifies the systemic hyperinflammatory response observed during sepsis and in sepsis RNA escaping from damaged tissues/cells may serve as an endogenous ligand for TLR3 thereby modulating immune responses [70].

Nucleic acids generated by infections or cell death have been detected within arteriosclerotic lesions and it is known that microbial and synthetic nucleic acids evoke inflammatory responses in cultured vascular cells. IFN-γ, a cytokine linked to atherosclerosis and graft arteriosclerosis, potentiated the inflammatory responses of intact arteries and cultured vascular smooth muscle cells (VSMCs) to poly (I:C) and was necessary for inflammatory responses of VSMC to self-RNA derived from autologous cells. IFN-γ induced the expression of TLR3, MDA5 e RIG-I. These findings from Ahmad et al. [71]

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suggest that exogenous or endogenous RNA sources may contribute to the inflammatory milieu of arteriosclerosis.

The study by Lee et al. [72] explored the possibility that Schwann cells are activated by endogenous TLR agonists released from damaged nerves and to examine this hypothesis, they tested whether Schwann cells were activated by necrotic neuron-derived molecules mediated by TLR2 and TLR3. Thus far, there are no in vivo data to support this hypothesis.

Baiersdörfer et al. [73] indicated that cellular RNA, following its release from necrotic rat cells in atherosclerotic lesions, can act as an endogenous TLR3 ligand to induce clustering (CLU—strong expression of CLU is associated with tissue regression, neurodegenerative conditions, malignant transformation, and cardiovascular diseases, such as myocarditis and atherosclerosis) expression in VSMC and in vivo. Thus, they expand the view on TLR2 and TLR4 as known pro-atherosclerotic effectors toward TLR3. Conclusively, TLR3 activation induces expression of cytoprotective and anti-inflammatory CLU by VSMC and mice, to potentially counteract atherosclerotic pathology.

A member of the noncoding mammalian transcripts has been identified. NTT (noncoding transcript in T cells) was found serendipitously during a search for genes that are differentially expressed among subsets of activated human $CD4^+$ T cells. This gene presents a 17-kb transcript and is selectively expressed in activated T cells [74]. NTT is probably involved with cell activation through IFNγ. Cells may employ different mechanisms to regulate RNA synthesis; in this context, it is noteworthy that the expression of the NTT gene could be implicated in the T lymphocyte response [75]. If there is an endogenous noncoding human RNA, a possible candidate would be the TLR3.

Although TLR3 recognizes viral double-stranded RNA, studies have suggested that host endogenous cellular mRNA released from either damaged tissue or contained within endocytosed cells may serve as a ligand for TLR3 (7). This may have important relevance in conditions such as sepsis, because RNA escaping from damaged tissues/cells may serve as an endogenous ligand for TLR3, thereby modulating immune responses.

TLR3 MODULATION—IMPLICATIONS FOR THERAPY

The extent and the quality of nucleoside modifications could alter the potency of RNA-mediated immune activation and be used as a means to differentiate between host and pathogenic RNA. It is known that TLRs are expressed not only on immune cells but also on cancer cells.

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A review by Karikó and Weissman [76] characterized a few naturally occurring nucleoside modifications of RNA and their influence on the capacity of RNA to activate immune cells and TLRs. RNAs containing modified nucleosides, and thus lacking immune-activating properties, have potential importance in clinical applications. Several studies have demonstrated that TLR3 activation by dsRNA directly inhibits cell proliferation and induces apoptosis in tumor cells [77–79]. In view of these promising effects, the use of dsRNA-derived compounds in combination with anticancer agents for chemo-immunotherapy warrants thorough investigation.

The RNA, probably, through secondary structure, is a potent activator of TLR3. TLR3 also responds to stimulation by cellular or in vitro transcribed RNAs. Although these are typically regarded as ssRNA, cellular and in vitro transcribed RNA can form secondary structures (hairpins) that contain double stranded sequences [7, 80, 81]. This finding has potential physiological relevance because RNA escaping from damaged tissue or contained within endocytosed cells could serve as an endogenous ligand for TLR3 that induces or otherwise modulates immune responses.

Hoffman et al. [69] suggest that self RNAs are capable of providing an innate immune stimulus that could predispose toward RNA binding protein–associated autoimmunity. This hypothesis, if found to extend to additional autoantigens, may substantially reshape our understanding of the role of innate immunity in the development and propagation of RNA binding protein–associated autoimmune syndromes.

RNA potently activated in vitro generated or freshly isolated human dendritic cells led to IL-12 and IFNα production and the enhanced expression of costimulatory molecules. These data will help guide the development of therapeutic applications of RNA, because avoiding modification may enhance the potency of immunotherapy involving RNA based vaccines or the use of RNA as an adjuvant. Conversely, appropriate nucleotide modifications could result in more efficient RNAi by dsRNA, including siRNA [82].

MicroRNAs play important regulatory roles in a variety of biological processes. In studies from Liu et al. [83], it was found that a microRNA, miR-147, was induced upon stimulation of multiple TLRs and functioned as a negative regulator of TLR-associated signaling events in murine macrophages. Expression of miR-147 was greater after cellular activation by TLR4 than after engagement of either TLR2 or TLR3, suggesting that maximal induction of miR-147 required activation of both NF-kB and IRF3. miR-147 mimics or the induced expression of miR-147 decreased, whereas miR-147 knockdown increased inflammatory cytokine expression in macrophages stimulated with ligands to

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TLR2, TLR3, and TLR4. These data demonstrate a negative-feedback loop in which TLR stimulation induces miR-147 to prevent excessive inflammatory responses.

Activation of TLR-3 inhibits human immunodeficiency virus (HIV) replication, but the mechanism(s) underlying the action of TLR-3 activation on HIV are unknown. Zhou et al. [84] demonstrated that treatment of monocyte-derived macrophages with poly I:C, significantly inhibited HIV infection and replication. Investigation of the mechanisms showed that TLR-3 activation resulted in the induction of type I interferon inducible antiviral factors, including APOBEC3G and tetherin, the newly identified anti-HIV cellular proteins. In addition, poly I:C-treated macrophages expressed increased levels of CC chemokines, the ligands for CCR5 and TLR-3 activation in macrophages induced the expression of cellular microRNAs. These findings indicate that TLR-3-mediated induction of multiple anti-HIV factors should be beneficial for the treatment of HIV disease where innate immune responses are compromised by the virus.

Poly (I:C) may produce toxic side effects in vivo, including shock, renal failure, coagulopathies, and hypersensitivity reactions [85]. A clinical grade, non-toxic analogue of poly (I:C) evaluated poly (I:C12U) (Ampligen) as a potential adjuvant for cancer immunotherapy because of its ability to drive maturation of human myeloid dendritic cells [86]. It has been verified that the human monocyte-derived dendritic cells primed with tumor lysate and matured with Ampligen are capable of generating Th1 specific anti-cancer responses in peripheral blood T-cells derived from cancer patients in the presence of ascites medium containing immunosuppressory cytokines. In summary, poly (I:C12U) appears to have potential for use as a clinical grade adjuvant for adoptive transfer or active immunization in cell-mediated cancer immunotherapy [87].

There is a series of RNA sensors in the innate immune system that discriminate self and non-self RNA on the basis of nucleoside modifications at 5′-end and internal regions of the RNA, although the molecular basis is probably different for each sensor. It appears that endosomal and cytosolic viral recognition pathways complement each other. Further discovery and characterization of RNA PAMPs will aid in understanding innate immunity and thereby provide the opportunity to make new and better RNA-based therapeutics [88].

Modification of poly (I:C) by the introduction of unpaired bases (uracil and guanine) results in unique "mismatched" dsRNAs capable of undergoing accelerated hydrolysis, and is associated with reduced toxicity in humans, without reduction in pharmacological activity. Interestingly, some differences have been reported on the functional

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activity of poly (I:C12U) with respect to poly (I:C) as maturation stimuli for dendritic cells [89], and on the signaling pathways activated. In fact, poly (I:C12U) signaling relies only on TLR3, while poly (I:C) activates both TLR3 and the cytosolic helicase MDA5 [90].

The double-stranded RNA (dsRNA) analogue poly (I:C) is a promising adjuvant for cancer vaccines because it activates both DCs and natural killer (NK) cells, concurrently promoting adaptive and innate anticancer responses. In these studies, McCartney et al. [91] investigated the relative contributions of MDA5 and TLR3 to poly (I:C)-mediated NK cell activation using MDA5(−), TLR3(−), and MDA5(−)TLR3(−) mice. MDA5 and TLR3 activated NK cells indirectly through accessory cells and induced the distinct stimulatory cytokines interferon-α and interleukin-12, respectively. Interestingly, multiple accessory cells were implicated, with MDA5 acting primarily in stromal cells and TLR3 predominantly in hematopoietic cells. Furthermore, poly (I:C)-mediated NK cell activation was not notably impaired in mice lacking CD8α DCs, providing further evidence that poly (I:C) acts through diverse accessory cells rather than solely through DCs. These results demonstrate distinct yet complementary roles for MDA5 and TLR3 in poly (I:C)-mediated NK cell activation.

Many adjuvants appear to be specific ligands for toll-like receptors (TLR) which are potent activators of innate immune responses. Some TLR agonists, such as CpG, have been shown to generate parallel immunosuppressive and inflammatory responses in innate immune cells capable of regulatory T cell induction and expansion. Various immunostimulant adjuvants are therefore under investigation in an effort to boost the immune system to overcome tolerance to tumor associated self-antigens and the ambient immunosuppressory influence of cytokines and regulatory T-cells [92].

It is known that TLRs are expressed not only on immune cells but also on cancer cells. Cancer cells also induce CXCL12 which recruits plasmacytoid dendritic cells that express CXCR4, the receptor of CXCL12, into the tumor microenviroment. Moreover, CXCL12 together with other factors, such as TNF α and IL-18, attract vascular dendritic cells to the tumor microenviroment, with a subsequent increase in tumor vascularization and metastasis [93]. Toll-like receptor 3 (TLR3) has gained recognition as a novel molecular target for cancer therapy because TLR3 activation by its synthetic ligand poly (I:C) directly causes tumor cell death [94]. TLR3 agonists can directly trigger apoptosis in human cancer cells and have been used as adjuvants to treat cancer patients with the aim of inducing an IFN-dependent immune response. Zhang et al. [95] examined the effect of TLR3 activation on

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the metastasis of nasopharyngeal carcinoma and reported that TLR3 activation downregulated the expression of chemokine receptor CXCR4 in a dose-dependent manner, and inhibited cell migration in response to CXCR4 ligand stromal cell-derived factor-1α (SDF-1α) in chemotaxis assays. TLR3 activation significantly reduced the capacity of nasopharyngeal carcinoma cells to form metastasis in draining lymph nodes when injected in athymic mice.

mRNA can be used as a vehicle for gene therapy because the immune system is naturally activated by foreign nucleic acids. Thanks to the presence of TLRs in endosomes, the delivery of foreign nucleic acids usually induces an immune response directed against the encoded protein. Meanwhile, the naturally transient and cytosolically active mRNA molecules are seen as a possibly safer and more potent alternative to DNA for gene vaccination. Optimized mRNA (improved for codon usage, stability, antigen-processing characteristics of the encoded protein, etc.) were demonstrated to be potent gene vaccination vehicles when delivered naked, in liposomes, coated on particles or transfected in dendritic cells in vitro. Human clinical trials indicate that the delivery of mRNA naked or transfected in dendritic cells induces the expected antigen-specific immune response. Follow-up efficacy studies are on the way. Meanwhile, mRNA can be produced in large amounts and GMP quality, allowing the further development of mRNA-based therapies [96].

The involvement of RNA and its receptors and the release of several cytokines and chemokines in the pathogenesis of immune disorders and other diseases, such as cancer, are still being studied and will have important implications in the future. Insights gained from study with endogenous RNA as noncoding regulatory RNAs could advance our understanding of various diseases where nucleic acids play a prominent role in the pathogenesis or regulation of gene expression, determine a role for nucleoside modifications on RNA, and give future directions for the design of therapeutic RNAs.

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Declaration of Interest

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

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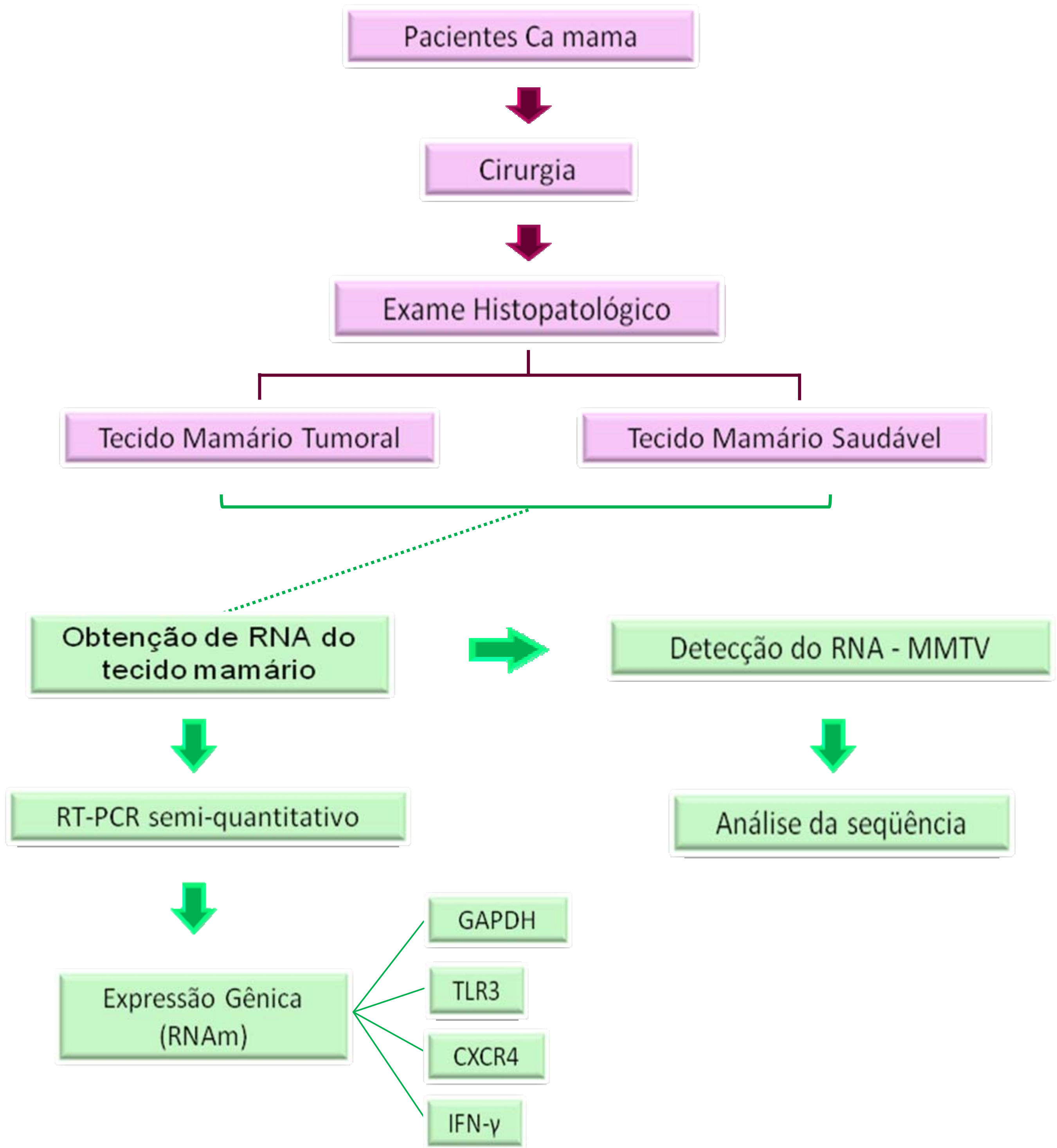
3.2 Receptor Toll-like 3: Possíveis Implicações no Microambiente do Câncer de Mama

O câncer de mama é uma doença complexa, heterogênea, cuja evolução depende da interação tumor-hospedeiro. O grau de heterogeneidade molecular e celular no câncer de mama e o grande número de eventos moleculares envolvidos no controle do crescimento celular, diferenciação, proliferação e metástases enfatizam a importância dos estudos acerca das alterações moleculares no câncer. É conhecido que TLRs são expressos não somente pelas células do sistema imune, mas também por células tumorais e que IFNγ pode ser uma citocina chave na resposta imune antitumoral. A próxima etapa do presente trabalho foi investigar a expressão de RNAm de TLR3, IFNγ e CXCR4 por PCR em tempo real, em tecido mamário tumoral das pacientes com câncer de mama (carcinoma ductal) comparado com tecido mamário saudável. A etiologia do câncer de mama tem sido muito discutida; variáveis como predisposição genética, idade e ambiente são comprovadamente fatores de risco, porem não são absolutos e únicos. Alguns vírus, devido a sua complexidade na estrutura e mecanismo de ação, são considerados agentes etiológicos de algumas neoplasias, sendo que o vírus do tumor mamário de camundongos (MMTV) tem sido sugerido como candidato a vírus causador de câncer de mama.

Delineamento Experimental II



Artigo 3

Toll-like Receptor 3: Implications for Proinflammatory Microenvironment in Human Breast Cancer

Abstract: Under many circumstances, the host constituents that are found in the tumor microenvironment support a malignancy network and provide the cancer cells with advantages in proliferation, invasiveness and metastasis establishment at remote organs. It is known that TLRs are expressed not only on immune cells but also on cancer cells and it has suggested a deleterious role for TLR3 in inflammatory disease. Hypothesizing that altered IFN signaling may be a key mechanism of immune dysfunction common to cancer as well CXCR4 is overexpressed among breast cancer patients, in the present study the mRNA expression of TLR-3, CXCR4 and IFNγ in breast cancer tumor tissues was investigated from women patients by real time PCR. The majority of the patients (92.3%; 25/26) were diagnosed with ductal carcinoma and a large number of patients included in this study presented stages II and III. No significant difference was observed when mRNA relative expression for TLR3 was assessed between healthy mammary and tumor tissue. However, when TLR3 mRNA expression was analyzed among different tumor stages and nodal status, it was observed that lymph node negative patients showed a significantly higher expression. It was also verified a positive correlation between mRNA relative expression of TLR3 and CXCR4 ($p<0.001$; rho=0.710) (Figure 3), and mRNA relative expression of TLR3 was significantly increased in breast cancer tumor tissue when compared to healthy mammary gland tissue among patients expressing high IFNγ, indicating that the proinflammatory microenvironment could lead to an up-regulation of CXCR4 mRNA and consequently to an increased TLR3 mRNA expression even among nodal negative patients. However a comprehensive study of TLR3, CXCR4 and IFNγ axis in primary breast tumors and corresponding healthy tissues will be crucial to further understanding of the cancer network.

Keywords: TLR3. IFNγ. CXCR4. Breast cancer.

Introduction

Toll like receptors (TLRs) are pattern recognition receptors for ligand molecules derived from microbes or host cells and TLR-ligand binding plays a key role in innate immunity and subsequent acquired immunity against microbial infection or tissue injury [1, 2].

TLR3 are expressed in the cytoplasm and can recognize viruses and respond to double-strand RNA. Damage-associated molecular patterns derived from injured healthy epithelial cells and necrotic cancer cells appear to be present at significant levels in the tumor microenvironment and their stimulation of specific TLRs can foster chronic inflammation [3].

Karikó & Weissman [4] characterized a few naturally occurring nucleoside modifications of RNA and their influence on the capacity of RNA to activate immune cells and TLRs. RNAs containing modified nucleosides, and thus lacking immune-activating properties, have potential importance in clinical applications. Several studies have demonstrated that the activation of TLR3 by dsRNA directly inhibits cell proliferation and induces apoptosis in tumor cells [5-7]. In view of these promising effects, the use of dsRNA-derived compounds in combination with anticancer agents for chemo-immunotherapy warrants thorough investigation.

TLR3 is expressed both intracellularly and on the cell surface of fibroblasts and epithelial cells, but is localized in the endosomal compartment of myeloid dendritic cells. Involvement of TLR3-TICAM-1 in activation of natural killer cells and cytotoxic T lymphocytes by myeloid dendritic cells suggests that TLR3 serves as an inducer of cellular immunity sensing viral infection rather than a simple IFN inducer [8].

It is known that the exposure of Kaposi's sarcoma cells (KS) to viral RNA ligands can result in a TLR3-mediated increase in the secretion of inflammatory proteins associated with KS cell growth that may contribute to disease [9].

Takahashi et al [10] demonstrated that nasal mucosa-derived fibroblasts express a large amount of TLR3 mRNA. TLR3 has also been implicated in the immunobiology of skeletal muscle. TLR3 is expressed in muscle cells both *in vitro* and *in vivo* and is upregulated by dsRNA and IFN. TLR3 levels were elevated in muscle biopsy specimens from patients with inflammatory and human immunodeficiency virus-associated myopathies, suggesting a deleterious role for TLR3 in inflammatory muscle disease [11].

Interferon - gamma (INFγ) coordinates a diverse array of cellular programs through transcriptional regulation of immunologically relevant genes. Cellular effects of IFN-γ are described, including up-regulation of pathogen recognition, antigen processing and presentation, the antiviral state, inhibition of cellular proliferation and effects on apoptosis, activation of microbicidal effector functions, immunomodulation, and leukocyte trafficking [12].

In breast cancer, the risk of metastatic disease is classically estimated by factors such as tumour size, tumour grade, estrogen and progesterone receptor status, ERBB2 (HER2/neu) overexpression and the number of positive

auxiliary lymph nodes (ALN). Numerous studies have shown that the presence of disseminated tumour cells in auxiliary lymph nodes is the most powerful prognostic factor and is associated with significantly poor disease-free and overall survival [13].

It was also demonstrated that samples of peripheral blood cells of stage II samples from breast cancer patients revealed higher CXCR4 expression than the controls and other stages [14]. CXC chemokine receptor 4 (CXCR4) plays various roles in many normal and pathological processes including embryogenesis, hematopoiesis, immunological homeostasis, human immunodeficiency virus infection, and the progression of rheumatoid arthritis [15].

In this study we proposed to investigate the expression and possible correlations among TLR3, CXCR4 and IFN gamma in the microenvironment of human breast cancer.

Methodology

Patients and Tumor Tissues

The protocol was approved by the institutional Human Research Ethics Committee of the State University of Londrina, Paraná, Brazil. The individuals with breast cancer were invited to participate, informed in detail regarding the research and voluntary written consent term was obtained from all of the patients enrolled. A term of free informed consent was signed by all sample donors and doctors involved prior to tissue collection. Samples of invasive breast carcinoma tissue and healthy mammary gland tissue from the same patient were obtained from a case series of 26 patients who had undergone surgery at the Londrina Cancer Institute, Parana State, Brazil. Clinical staging was determined according to the Union of International Control of Cancer (UICC) classification criteria. Healthy breast tissue was obtained from the adjacent tissue of patients undergoing surgery for the tumors.

RNA isolation and reverse transcriptase reaction

Total cellular RNA was extracted from tissue cells with TRIzol LS reagent (Invitrogen^{TM}, Carlsbad, California, USA) according to the manufacturer's

instructions. Purified total RNA was quantified and assessed for purity by determining absorbance at 260 and 280 nm and was then stored at –80°C until testing. Reverse transcriptase reaction was performed using 500ng RNA, 20 units cloned Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT; Invitrogen™), 4 units Recombinant Ribonuclease Inhibitor (RNaseOUT™; Invitrogen™) under the following conditions: 2.5µM oligo dT, 50mM Tris HCl pH 8.3, 75mM KCl, 1.5mM $MgCl_2$, 1.25mM dNTP, at 42°C for 60 min in a Biocycler (Biosystems, Guelph, Ontario, Canada).

Molecular analysis of Beta-actin Mrna

PCR for beta-actin cDNA was determined as described by Amarante et al [16]. Briefly, cDNA synthesis was carried as previously described and the PCR conditions were: 94ºC for 1 min followed by 35 cycles at 94ºC for 30 sec, 55ºC for 30 sec, 72ºC for 1 min and finally, 72ºC for 10 min in a Biocycler (Biosystems, Guelph, Ontario, Canada). PCR products were analyzed by electrophoresis on acrylamide gel (10%) and detected by a nonradioisotopic technique using a commercially available silver staining method.

Quantitative real-time PCR for TLR3, CXCR4 and IFNγ mRNA

Real-time PCR using SYBR green fluorescence was performed with 20 g cDNA in a total volume of 20µL. Quantitative real-time PCR reaction was carried out using Platinum$^{®}$SYBR Green qPCR SuperMix UDG (Invitrogen ^{TM}) with 0.25 M of each sense and antisense primers **(Table I)**. The PCR reaction was performed for 40 cycles as follows: 95°C for 30 sec, 54ºC for 30 sec and 72°C for 30 sec in a Chromo4™ Real Time PCR Detection (Bio-Rad, Hercules, USA). In the quantitative RT-PCR analysis the expression level of mRNA was calculated according to the Pfaffl method [17], in which Ct values for the target gene were the mean fold change + SEM for three independent determinations corrected by human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) Ct values from control samples, considering efficiency values.

Table 1 – Quantitative real-time PCR Conditions.

Gene Mrna	GenBank Acession Number	Primer	Sequence
CXCR4	AF025375	*Foward*	5' TCTACTCCATCATCTTCTTTA 3'
		Reverse	5' ACGTTGGCAAAGATGAAGGTC 3'
IFNγ	NM_000619	*Foward*	5' AATTGTCTCCTTTTACTTCA 3'
		Reverse	5' GTCATCTCGTTTCTTTTTGT 3'
GAPDH	NM_002046	*Foward*	5' GAAGGTGAAGGTCGGA 3'
		Reverse	5' GGGTCATTGATGGCAAC 3'
TLR3	NM_003265	*Foward*	5' AAATAGACAGACAGACAGAACAGT 3'
		Reverse	5' AAAAACACCCGCCTCAAA 3'

Statistical Analysis

Statistical analyses were conducted using the SPSS Statistics 17.0 program (SPSS inc., Chicago, Illinois, USA). A *p* value ≤0.05 indicated statistical significance. The Kruskal Wallis Test was used to check expression and the two-tailed Spearman's rank analysis was used to analyze correlation for TLR3, IFNγ and CXCR4 expression.

Results

In the present study, the expression of TLR3 mRNA, CXCR4 mRNA and INFγ mRNA in breast cancer tumor tissue was investigated in 26 women, aged 40 to 86 years old, average age 60 years, attended at the Londrina Cancer Institute, Parana, Brazil.

The majority of the patients (92.3%; 25/26) were diagnosed with ductal carcinoma, according to the clinical criteria determined by the Union of International Control of Cancer (UICC, 1958) [18] (**Table II**). A large number of patients included in this study presented stages II and III (69.23%; 18/26), while the number of patients who presented stages I and IV was relatively small (15.38%; 4/26).

Table 2 – Clinicopathological features of breast cancer patients (n=26).

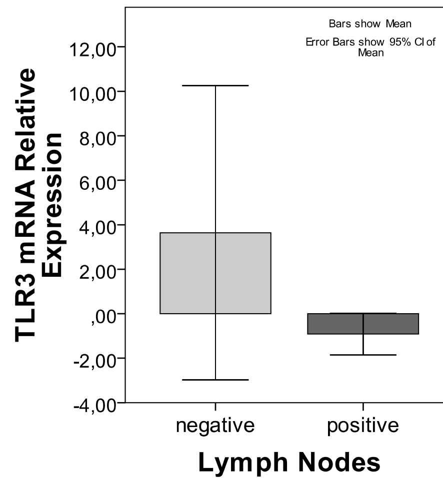
Age (years)	< 40	1 (3.85)
	41 – 50	5 (19.23)
	51 – 60	8 (30.77)
	> 60	12 (46.15)
Tumor Stage	I	3(11.54)
	II	16(61.54)
	III	6(23.07)
	IV	1(3.85)
Tumor Histology *	IDC	25 (96.15)
	ILC	1 (3.85)
Nodal Status	Negative	8 (30.77)
	Positive	8 (30.77)
	unknown	10 (38.45)

*IDC – Invasive Ductal Carcinoma
*ILC – Invasive Lobular Carcinoma

Before the TLR3 mRNA assays, the viability of the RNA samples and cDNA quality were analyzed by conventional PCR for beta-actin, performed with specific primers. When occur contaminants of genomic DNA, the amplification product would be to 573bp. No contamination with genomic DNA was observed, since all the amplified products presented a fragment correspondent a 353bp. All the RNA samples presented detectable quantities of beta-actin mRNA and acceptable integrity during amplification.

In the next step, the relative expression of TLR3, INFγ and CXCR4 was determined comparing healthy mammary tissue with tumor mammary tissue for the same breast cancer patient using the Pfaffl method. TLR3 RNAm levels were evaluated using real time PCR. No significant difference was observed when mRNA relative expression for TLR3 was assessed between healthy mammary and tumor tissue. However, when TLR3 mRNA relative expression was analyzed among different tumor stages and nodal status, it was observed that lymph node negative patients showed a significantly higher expression ($p = 0.013$) (**Figure 1**).

Figure 1 – TLR3 mRNA relative expression according to nodal status. Correlations were evaluated by 2-tailed Spearman's rank. (p= 0.013). Bars show mean and error bars show 95% CI of mean.



TLR3 mRNA relative expression was significantly correlated to IFNγ mRNA relative expression (p=0.001; rho=0.612), as shown in **Figure 2,** and mRNA relative expression TLR3 was significantly correlated to CXCR4 (p<0.001; rho=0.710), as shown in **Figure 3**.

Figure 2 – Correlation among TLR3 and IFNγ expression. Correlations were evaluated by 2-tailed Spearman's rank. (p=0.001, rho = 0.612).

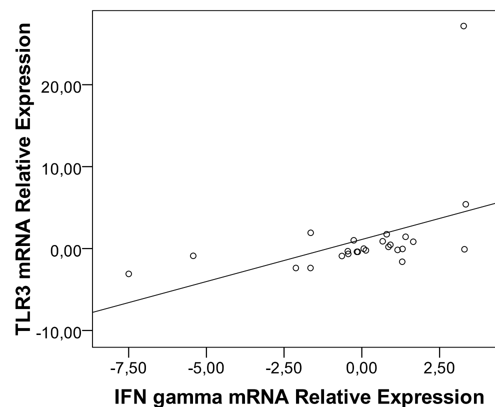
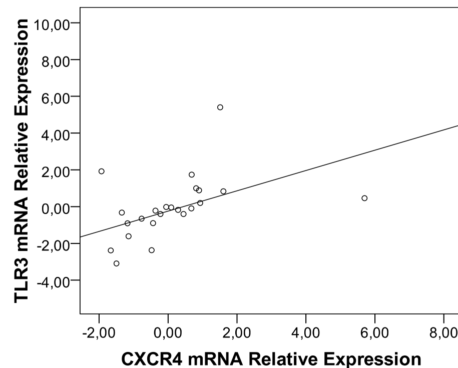


Figure 3 – Correlation among TLR3 and CXCR4 mRNA expression. Correlations were evaluated by 2-tailed Spearman's rank. (p<0.001, rho = 0.710).



Discussion

Clinicopathological parameters have been validated and serve as a guide for the use of systemic therapy and prognostication. These include tumor size, lymph node stage and histological grade, histological type and the patients' age [19], molecular profile and response to therapy [20].

The incidence and prevalence of most cancers increase with age [21] and our results are in agreement. In the present study, the age range of 26 women breast cancer patients was 40 to 86 years old, the average age was 60. The association between cancer and age can be explained by a more prolonged exposure to carcinogens in older individuals, what would lead to age-associated tissue dysfunction caused by the accumulation of molecular and cellular damage [22] since aging is associated with the inability to maintain and repair somatic cells [23].

Many TLR3 effects rely on cells of the innate immune system that either express TLR3 or respond to inflammatory mediators that are produced upon TLR3 signaling. Immune cells that express TLR3 and contribute to an innate immune response are dendritic cells, macrophages, natural killer cells, and mast cells [24-26].

Under many circumstances, the host constituents that are found in the tumor milieu support malignancy cascades and provide the cancer cells with advantages in proliferation, oxygen and nutrient supply, invasiveness, and metastasis establishment at remote organs [27]. TLR3 mRNA relative expression was analyzed among different tumor stages and nodal status and it was observed that lymph node negative patients presented a significantly higher expression.

Research by Tabiasco et al [28] demonstrated that TLR3 is also present in cells that participate directly in the adaptive immune response in which human effector CD8$^+$ T lymphocytes express TLR3 as a functional coreceptor. In this context, TLR3 ligation was shown to directly increase IFNγ production by antigen-primed CD8$^+$ T cells. This evidence indicated that TLR3 is a “danger” receptor with a pleiotropic potential in innate and adaptive immunity [29]. These authors have demonstrated that many reports show that TLR3 contributes to the elimination of specific viruses, but others demonstrate that some viruses can benefit from TLR3 stimulation. The general outcome is probably dependent on several factors, such as the type of virus, the viral load, its infection mode (endoplasmic versus cytoplasmic), the cell type that is infected, and the stage of infection.

In this study, IFNγ expression presented no correlation to clinopathological features, but a significantly higher IFNγ expression was observed in breast tumor tissues from stage II patients (p=0.014) (data not shown). Interestingly, this increase was followed by an increase in TLR3 in the same patients.

It has been proposed that efficient IFN signaling is critical to lymphocyte function; animals rendered deficient in peripheral IFN signaling develop cancer at higher rates and impaired-IFN signaling was equally evident in stage II, III, and IV breast cancer patient periphery blood, suggesting that altered IFN signaling may be a key mechanism of immune dysfunction common to cancer [30].

Results from Negishi et al [31] bring the TLR3-type II IFN axis to the forefront of our understanding of the host’s antiviral innate immune response. In their hypothetical model, TLR3 mediates the production of type II IFN, which then functions in parallel with the type I IFN system that is elicited by RIG-I/MDA5 cytosolic receptors. The TLR3-type II IFN axis is sufficient to reduce Coxsackievirus group B serotype 3 (CVB3- a member of the positive-stranded RNA virus family *picornaviridae*) replication systemically and, at the same time, to prevent local tissue damage. Thus, these two arms of innate immunity presumably exert their functions by coupling with each other to mount a full-blown antiviral response.

Although the presence of lymph node (LN) metastasis is a negative prognostic factor for breast cancer and other cancers, it is not yet possible to reliably identify those patients who will eventually relapse with metastatic disease only from their LN status at primary therapy, indicating that other ways of metastatic tumor cell

spread also play an important role [32]. Considering that significant numbers of LN-negative patients develop metastatic disease, the reliability of current staging procedures to detect DTC in LN has been questioned [19].

Data from McCall et al [33] indicated that papillary thyroid carcinoma cells basally express TLR3 and TLR3 signal systems are functional in these cells. High basal TLR3 levels and TLR3 signals are capable of increasing cytokines.

Kato et al [34] reported that the CXCR4 expression pattern was significantly correlated with the degree of lymph node metastasis in breast cancers and Rhodes et al [35] reported that CXCR4 overexpression is indeed correlated with worse prognosis and decreased patient survival irrespective of the status of the estrogen receptor (ER).

In the present study there was no statistically significant differences in the expression of CXCR4 mRNA, IFNγ and TLR3 between healthy and tumor tissues, however, it was observed a positive correlation between mRNA relative expression of TLR3 and CXCR4, and mRNA relative expression of TLR3 was significantly increased in breast cancer tumor tissue when compared to healthy mammary gland tissue among patients expressing high IFNγ.

Since the tumor microenvironment plays important roles in cancer initiation, growth, progression, invasion and metastasis [36], it is possible to propose that an overexpression of IFNγ mRNA due to the proinflammatory microenvironment can lead to an up-regulation of CXCR4 mRNA and consequently to an increased TLR3 mRNA expression even among nodal negative patients.

Although the comprehensive study of TLR3, CXCR4 and IFNγ axis in primary breast tumors and corresponding normal tissues will be crucial to further understanding of the cancer network, the present study suggests that TLR3, CXCR4 and IFNγ has important implications in the immunopathogenesis of breast cancer.

Conflict of Interest Statement

The authors declare to comply with all requirements for publication there are no conflict of interest.

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REVIEW

The possible involvement of virus in breast cancer

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Abstract It is well known that the etiology of human breast cancer is significantly affected by environmental factors. Virus-associated cancer refers to a cancer where viral infection results in the malignant transformation of the host's infected cells. Human papillomaviruses (HPV), mouse mammary tumor virus (MMTV) and Epstein–Barr (EBV) virus are prime candidate viruses as agents of human breast cancer. The precise role that viruses play in tumorigenesis is not clear, but it seems that they are responsible for causing only one in a series of steps required for cancer development. The idea that a virus could cause breast cancer has been investigated for quite some time, even though breast cancer could be a hereditary disease; however, hereditary breast cancer is estimated to account for a small percentage of all breast cancer cases. Based on current research, this review present at moment, substantial, but not conclusive, evidence that HPV, EBV and MMTV may be involved in breast cancer.

Keywords Breast cancer · MMTV · HPV · EBV

Introduction

Several studies suggest viral oncogenesis as an etiological factor for breast cancer, but it remains controversial. Many risk factors have been associated with the pathogenesis of this disease. However, the molecular events in the genesis of most breast cancers are unclear (Dimmock and Primrose 1994). The involvement of viruses in certain breast tumors and cells lines has been described. Magrath and Bhatia (1999) and Arbach et al. (2006) described a role for Epstein–Barr virus (EBV) in breast cancer and Wong et al. (2002) studied new associations of human papillomavirus, Simian virus 40, and EBV with human cancer.

The purpose of a study by Trabelsi et al. (2008) was to detect EBV in breast cancer. Positivity was observed in tumor cells, but not in nontumoral epithelial cells nor in lymphoid cells, suggesting a possible implication of EBV in these tumors, although further studies are required.

Lymphoepithelioma-like carcinoma (LELC) of the breast is a rare tumor, with few cases documented in the literature. Every reported case was EBV negative and no other viral etiology was suggested. Lymphoepithelioma-like carcinomas are not well circumscribed; they show either Schminke's or Rigaud's growth patterns or both, they can be estrogen receptor positive and, in contrast to medullary carcinomas, the stromal infiltrate contains few plasma cells. In 2005, Kurose et al. reported that LELC of the breast shows evident ultrastructural glandular differentiation. LELC are tumors with morphologic features identical to those of undifferentiated nasopharingeal carcinoma. The presence of HPV has been verified in LELC carcinoma of the breast (Kulka et al. 2008), but some authors have reported negative cases for EBV (Dadmanesh et al. 2001; Ilvan et al. 2004; Sanati et al. 2004).

Studies involving human gestational breast cancer that arises during or immediately after pregnancy reveal that it is associated with a poorer prognosis than other types of breast cancer (Wang et al. 2003). One possible interpretation of these data is that the expression of mouse mammary

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tumor virus-like sequences present in the human genome is stimulated by pregnancy hormones via the hormone response element (HRE), leading to increased viral load and reinfection. Indeed, the same authors also reported MMTV-specific RNA expression in human mammary tumors carrying such MMTV-like sequences (Wang et al. 1998).

Wang et al. (1998) identified mouse mammary tumor virus (MMTV) mRNA in breast cancer specimens, but not in normal breast tissues. Indik et al. (2007) demonstrated that MMTV rapidly spreads in cultured human breast cells, ultimately leading to the infection of all the cells in culture, thus providing further evidence that human cells are compatible hosts for MMTV. Their observations further suggest that cross-species transmission of MMTV is generally possible and strengthens the contention that MMTV might be an etiological agent involved in human breast carcinogenesis.

Viral particles isolated from primary cultures of human breast cancer cells have been characterized. The proviral structure of a retrovirus has been described in human breast cancer. This provirus, designated as human mammary tumor virus (HMTV), was 95% homologous to MMTV and revealed features of a replication-competent virus (Melana et al. 2007).

Human papillomaviruses (HPVs) and mouse mammary tumor virus (MMTV) possess hormone responsive elements that appear to be associated with enhanced replication of these viruses in the presence of corticosteroid and other hormones. Viral genetic material for each of these candidate viruses has been identified by polymerase chain reaction (PCR) in breast tumors, but rarely in normal breast tissue controls. Pooled data from controlled studies show substantial odds ratios for the presence of viral genetic material in breast tumors compared with normal controls. These and additional data provide substantial, but not conclusive, evidence that HPV, MMTV and EBV may play a role in the etiology of human breast cancer (Lawson et al. 2006a, b).

Within this context and based on current research, the present review investigates the possible involvement of viruses such as Epstein–Barr virus, human papillomaviruses and mouse mammary tumor virus on breast cancer.

Epstein–Barr virus (EBV) and breast cancer

For many years, correlations between EBV replication and the appearance of a malignant phenotype were limited to nasopharyngeal carcinoma and lymphoid cells. Controversy regarding the association of EBV, a ubiquitous human herpesvirus, with breast cancers has been reported in the literature. A hypothesis was proposed that primary EBV infection occurring during adolescence or adulthood could be associated with elevated breast cancer risk (Yasui et al. 2001).

EBV might have a role at an early step in carcinogenesis and then be lost after the development of the tumor. Alternatively, infection with EBV at a late state of tumor development might enhance oncogenic properties, such as invasiveness, angiogenesis and metastasis (Murono et al. 2001; Wakisaka 2002). These findings raise the possibility that EBV might alter the phenotype of a subpopulation of carcinomatous cells so that they become more aggressive in behavior (Wakisaka and Pagano 2003).

This cancer is very frequent and the involvement of EBV in even a small proportion of breast cancers could have important implications (Trabelsi et al. 2008). The role of EBV in the pathogenesis of breast cancer has been of longstanding interest to the field. Breast epithelial cells can be infected by EBV through direct contact with EBV-bearing lymphoblastoid cells and EBV infection has recently been shown to confer increased resistance to chemotherapeutic drugs in breast cancer cells (Lin et al. 2007).

Perkins et al. (2006) proposed that the detectable levels of EBV DNA found in the tumor and the absence or lower amounts of viral DNA found in matched peripheral blood support a relationship between Epstein–Barr virus and breast carcinoma. Lin et al. (2007) established EBV-infected breast cancer MCF7 and BT474 cells and demonstrated that EBV infection promotes tumorigenic activity of breast cancer cells.

Trabelsi et al. (2008) suggested a possible implication of EBV in two types of breast cancer: medullary carcinoma and high grade invasive ductal carcinoma with lymphoid stroma, although further studies along these research lines are required. Several laboratories have reported the detection of EBV in a subset of breast tumors (Bonnet et al. 1999; Fina et al. 2001; Labrecque et al. 1995; Luqmani and Shousha 1995; Murray et al. 2003); however, negative results have also been reported (Chu et al. 2001; Deshpande et al. 2002; Herrmann and Niedobitek 2003). Nevertheless, in most of the studies available, a low viral load was detected in breast cancer biopsy specimens and infected cells were not clearly identified. Through microdissection and isolation of pure tumor cells, the findings were that even in EBV-positive tumor samples, many tumor cells do not contain EBV genomes and that breast carcinomas are highly heterogeneous in terms of genome content and distribution. Such findings raise the possibility that although EBV is unlikely to present an etiological role in the genesis of breast cancer, the virus might contribute to tumor progression. Consequently, if even a small number of breast cancer cells are EBV infected, the impact of EBV infection on the efficiency of anticancer treatment might be of clinical importance (Arbach et al. 2006).

Genes are expressed by many human tumors of different histological types but not by normal cells, except for male germline cells. In this context, Hennard et al. (2006) reported that one of the antibodies frequently employed to detect nuclear antigen 1 (EBNA 1) in tissue samples cross-reacts with the MAGE4 protein, a cancer antigen expressed in many cancer types. Their observation suggests that reports documenting an EBV association on the basis of reactivity with this antibody must be considered unreliable.

Ribeiro-Silva (2005) demonstrated the presence and expression of EBV restricted to epithelial tumor cells in a subset of breast carcinomas in Argentine patients. The pathophysiological significance and clinical implications of EBV presence in breast carcinomas remain unclear, but the notion of a silent passenger for this "carcinogenic virus" was discarded.

Table 1 shows over 25 studies of EBV and breast cancer tissues using molecular biology involving methodology.

Human papillomaviruses (HPV) and breast cancer

Cervical cancer development is a multistep process. The major steps are HPV infection and HPV persistence for over 1 year, followed by slow progression to precancerous lesions and, eventually, to invasive cancer. Most HPV infections spontaneously resolve in 6–12 months and the majority of precancerous lesions regress due to immune response. Since only a small proportion of HPV infections will eventually lead to cervical cancer, other cofactors are required for cervical cancer development (Schiffman and Kjaer 2003). It is well established that high-risk HPVs are the major cause of cervical cancer. HPV is the most prevalent sexually transmitted viral infection among men and women and it is estimated that 80% of sexually active adults have been infected with at least one HPV type (Baseman and Koustsky 2005).

Two major classes of genital HPV types have been identified according to their association with cervical cancer.

Table 1 Results of studies into the presence of EBV genetic material in human breast cancer

Region	Sample	Controls/EBV+ (%)	Cases	EBV+ (%)	Authors
USA	Paraffin-embedded tissue	–	35	0.0	Gaffey et al. (1993)
Japan	Paraffin-embedded tissue	–	03	66.0	Horiuchi et al. (1994)
UK	Frozen	21/(0.0%)	91	21	Labrecque et al. (1995)
Belgium	Paraffin-embedded tissue	–	10	0.0	Lespagnard et al. (1995)
UK	Paraffin-embedded tissue	–	28	54.0	Luqmani and Shousha (1995)
Taiwan	Paraffin-embedded tissue	–	60	0.0	Chu et al. (1998)
USA	Paraffin-embedded tissue	–	107	0.0	Glaser et al. (1998)
France/UK	Frozen	30/(10.0%)	100	51.0	Bonnet et al. (1999)
Holland	Frozen	–	24	21.0	Brink et al. (2000)
Japan	Paraffin-embedded tissue	–	61	0.0	Kijima et al. (2001)
USA	Frozen/Paraffin-embedded tissue		48	10.42	Chu et al. (2001)
North Africa/Southern France/Northern Europe	Frozen/Paraffin-embedded tissue	03/(0.0%)	509	31.8	Fina et al. (2001)
USA	Frozen	–	115	2.0	McCall et al. (2001)
USA	Paraffin-embedded tissue	21/(0.0%)	33	42.0	Grinstein et al. (2002)
USA	Paraffin-embedded tissue	11/(0.0%)	20	45.0	Kleer et al. (2002)
USA	Paraffin-embedded tissue	–	43	0.0	Deshpande et al. (2002)
UK	Paraffin-embedded tissue	–	92	21.0	Murray et al. (2003)
Germany	Paraffin-embedded tissue	–	59	6.8	Herrmann and Niedobitek (2003)
UK	Fresh Frozen	–	15	40.0	Xue et al. (2003)
USA	Paraffin-embedded tissue	–	11	36.36	Lau et al. (2003)
USA	Paraffin-embedded tissue	–	55	7.0	Thorne et al. (2005)
Argentina	Fresh tissue	48/0.0%)	39	31.0	Preciado et al. (2005)
USA	Paraffin-embedded tissue	45/(0.0%)	45	0.0	Perrigoue et al. (2005)
Turkey	Paraffin-embedded tissue	54/(35%)	57	22.81	Kalkan et al. (2005)
Taiwan	Frozen	60/(0.0%)	62	45.2	Tsai et al. (2005)
France	Frozen	–	95	46	Arbach et al. (2006)
USA	Fresh Frozen	–	24	45.8	Perkins et al. (2006)
France	Paraffin-embedded tissue	–	36	13.89	Trabelsi et al. (2008)
Egypt	Paraffin-embedded tissue	20/(0.0%)	40	25.0	Fawzy et al. (2008)

The low-risk types, especially HPV 6 and HPV 11, are almost never isolated in cervical malignancies. In contrast, viral DNAs from the high-risk types are identified in most cervical cancer cases; although the vast majority of lesions in which they are found are nonmalignant. HPV16 and HPV18 are the two most carcinogenic HPV types and are responsible for 70% of cervical cancer and about 50% of cervical intraepithelial neoplasia (CIN) grade 3 (CIN3) (Smith et al. 2007).

Di Lonardo et al. (1992) were the first to report the relationship between HPV and breast cancer, demonstrating HPV-16 DNA in 29.4% of 17 breast carcinoma samples and it has been proposed that HPV type 16 is present in many invasive and metastatic breast cancers and less frequently in situ breast cancer (Yasmeen et al. 2007). It has been suggested that high-risk HPV infection can induce cell invasion and metastasis in breast cancer through Id-1, a family of helix–loop–helix transcription factors (Yasmeen et al. 2007; Akil et al. 2008).

The identification of HPV by de Villiers et al. (2005) Damin et al. (2004) and Kan et al. (2005) in breast tumors has established HPV as strong candidate oncoviruses for breast cancer. Widschwendter et al. (2004) detected HPV DNA in breast cancer tissues of patients presenting cervical cancer history.

Cervical cancer is cancer of the uterine cervix and is a common cause of death among middle-aged women (40–60 years old). Several studies detected different HPV types in breast carcinomas that were members of the high risk group, such as HPV-16, HPV-18 and HPV-33 described in invasive ductal carcinomas (de Villiers et al. 2005). Kroupis et al. (2006) verified the presence of high-risk HPV sequences in breast cancer tissues and in association with histopathological characteristics.

Fluorescence in situ hybridization with an HPV-33 DNA probe proved the presence of the HPV-33 genome in a few tumor cell nuclei. Therefore, Kulka et al. (2008) suggested that the tumor cells themselves contain the HPV genome. The morphology of tumor cell nuclei also supports this hypothesis. These authors proposed international collaboration to further study lymphoepithelioma-like carcinoma (LELC) cases of the breast, including the presence or absence of previous history of cervical carcinoma, the presence or absence of HPV in the tumor, together with all follow-up data, which they believed would rapidly increase current knowledge of this rare type of breast carcinoma.

Although researchers have described the presence of HPV in breast cancer patients, no HPV-DNA sequences were detected in samples using DNA amplification by PCR to detect papillomavirus DNA. These data reported by de Cremoux et al. (2008) argue against the role of oncogenic HPV in the pathogenesis of breast cancer.

Table 2 shows studies involving molecular biology for HPV detection in breast cancer tissues.

Table 2 Results of studies into the presence of HPV genetic material in human breast cancer

Region	Sample	Controls/HPV+ (%)	Cases	HPV+ %	Authors
Italy	Paraffin-embedded tissue	–	17	29.41	Di Lonardo et al. (1992)
USA		–	13	0.0	Bratthauer et al. (1992)
UK		–	80	0.0	Wrede et al. (1992)
India	Fresh	–	30	0.0	Gopalkrishna et al. (1996)
China and Japan	Paraffin-embedded tissue	–	72	41.67 11.11	Yu et al. (1999)
Norway	Paraffin-embedded tissue	–	41	46.34	Henning et al. (1999)
USA	Fresh tissue	–	17	35.29	Liu et al. (2001)
China	Paraffin-embedded tissue	–	28	23.17	Li et al. (2002)
Brazil	Paraffin-embedded tissue	41/(0.0%)	101	24.75	Damin et al. (2004)
Taiwan	Fresh tissue	60/(0.0%)	69	12.9	Tsai et al. (2005)
Austria	Fixed	–	11	63.64	Widschwendter et al. (2004)
Germany	Paraffin-embedded tissue	–	29	86.0	de Villiers et al. (2005)
Australian	Paraffin-embedded tissue	–	50	48.0	Kan et al. (2005)
Greece	Frozen tissue	–	107	15.9	Kroupis et al. (2006)
Turkey	Fresh tissue	16/(32%)	50	74.0	Gumus et al. (2006)
Korea	Paraffin-embedded tissue	–	123	6.5	Choi et al. (2007)
Swiss	Paraffin-embedded tissue	–	81	0.0	Lindel et al. (2007)
Mexico	Paraffin-embedded tissue	40/(0.0%)	67	4.47	Mendizabal-Ruiz et al. (2008)
Japan		11/(0.0%)	124	21.0	Khan et al. (2008)
France	Fresh Tissue	–	50	0.0	de Cremoux et al. (2008)

Mouse mammary tumor virus (MMTV) and breast cancer

Mouse mammary tumor (MMTV) virus has been used as a model for the study of breast cancer since its discovery in the 1920s as a milk-transmitted agent. The MMTV infection cycle has been reported and the contribution of virus-encoded proteins to mammary tissue transformation (Ross 2008).

Although MMTV, the prototype β-retrovirus, was discovered by Bittner more than half a century ago (Bittner 1936), the biology of this virus is still not completely understood mainly due to difficulties in obtaining high titers of the virus in cell culture, its poor infectivity compared with γ-retroviruses and the lack of a simple and sensitive in vitro assay to evaluate the infectivity and transforming potential of the virus (Indik et al. 2005).

MMTV, a member of the betaretroviridae, is the most common cause of breast cancer and T cell lymphomas in mice and is transmitted in mice in the germline, as endogenous proviruses, and exogenously, as infectious virions. MMTV spreads like a cold virus from person to person, although researchers are not certain whether this virus spreads by sneezing, food contamination or some other means of transmission. The involvement of MMTV with human pathogenesis was based on immunological and molecular biology evidence and was proposed some time ago (Sarkar 1980).

MMTV, starting with the infection and activation of dendritic cells and B cells that leads to the expression of a viral superantigen followed by professional superantigen-mediated priming of naive polyclonal T cells by dendritic cells and induction of superantigen-mediated T cell B cell collaboration results in long-lasting germinal center formation and production of long-lived B cells that can later carry the virus to the mammary gland epithelium. Later in life it can induce mammary gland transformation by integrating close to proto-oncogenes leading to their overexpression (Acha-Orbea et al. 2007).

The hypothesis that a retrovirus homologous to the mouse mammary tumor virus is involved in human breast cancer an etiology has fascinated scientists from many years, but it has never been convincingly demonstrated. Renewed interest in this hypothesis developed when an MMTV env gene-like sequence was found in 38% of human breast cancer tissues. Whereas some subsequent studies confirmed these findings, others did not. The main reasons for this discrepancy included the different sensitivities and technical details of current molecular approaches to the detection of these sequences (Zammarchi et al. 2006).

Previous studies have found signs of the virus in breast cancer tissue taken from women. Szabo et al. (2005) reviewed the observation that a subset of cats infected with a close homologue of MMTV could be of epidemiological significance for human breast cancer. Cats can become infected by MMTV from mice and, in turn, may transmit the virus to humans, possibly after selection for variants with an expanded host range.

Witt et al. (2003) reported that the MMTV-like env gene sequence was not detectable in breast cancer tissue of Austrian patients; similarly, studies by Zangen et al. (2002) could not confirm a role for MMTV-like env gene as a molecular marker for breast cancer. The detection of MMTV-like env sequences has been reported in variable proportions that did not exceed 40% of BC cases in several countries. However, these viral sequences were found in higher proportion (74%) in Tunisian women diagnosed with BC during the 1970s (Hachana et al. 2008).

MMTV-like env gene sequences, which indicate the presence of a replication-competent MMTV-like virus, have been identified in some human breast cancers, but rarely in normal breast tissues. However, no evidence for a causal role of an MMTV-like virus in human breast cancer has emerged, although there are precedents for associations between specific histological characteristics of human cancers and the presence of oncogenic viruses (Lawson et al. 2006a, b).

Varied MMTV-like envelope gene (env) sequences have been identified in up to 74% of human breast cancers. However, the role and origin of these MMTV-like sequences in human breast cancer remain uncertain. Mok et al. (2008) studied the integration of MMTV-like env sequences in human breast cancer. PCR screening has identified 28 (56%) Australian breast cancer specimens and 7 (87.5%) human breast cancer cell lines to be positive for the MMTV-like env sequence.

It is known that human endogenous retroviruses (HERVs) account for up to 9% of the human genome and include more than 800 elements related to betaretroviruses. While MMTV is the accepted etiological agent of mammary tumors in mice, the role of retroviral elements in human breast cancer remains elusive. In a study by Frank et al. (2008), no evidence for MMTV or human MMTV-like virus transcripts was found, indicating that transcriptionally active, MMTV analogous, exogenous viruses were not present in the breast cancer samples analyzed.

Despite the widely accepted belief that human cells are not appropriate hosts for MMTV, results from Indik et al. (2007) show that human cells can support replication of mouse mammary tumor virus.

MMTV-like gene sequences were amplified in the lung cancer cell INER-51, but not in the MCF-7 cell line that has been used as a positive control in other reports and in five out of 119 (4.2%) breast cancer biopsy tissues. Furthermore, the identity of sequences of PCR products from INER-51 and breast cancer-positive samples are 98 and 99% when compared with the env region of MMTV. These results indicate that MMTV-like gene sequences are present in the Mexican population (Zapata-Benavides et al. 2007).

Melana et al. (2007) described the complete proviral structure of a retrovirus in human breast cancer. This provirus, designated as human mammary tumor virus (HMTV), was 95% homologous to MMTV and revealed features of a replication-competent virus. It has been shown that breast cancer cells in primary cultures produced HMTV viral particles that are similar to the mouse virus and may play a role in human breast cancer pathogenesis.

Despite the high sensitivity of the real-time PCR method used, none of the samples were positive for HTMV DNA or RNA. The absence of HTMV in both breast cancer samples and controls indicates either that the concentration of putative HMTV DNA in the breast cancers was too low for detection or that it did not exist there (Bindra et al. 2007).

A study was reported involving three members of the same family, father, mother, and daughter, who were diagnosed with carcinoma of the breast with axillary nodal metastases. The father was the first to be diagnosed at the age of 79 in 1963. The mother and daughter were each diagnosed six years later in 1969 at the ages of 82 and 56, respectively. All three family members had invasive carcinoma (Etkind et al. 2008).

The possibility of using the MMTV variants as alternative models for analyzing mammary tumor stem cells and pregnancy-associated breast cancer in women was discussed by Kordon (2008).

A MMTV-like long terminal repeat superantigen in human breast cancer has been sequenced from human breast cancer and has been found highly homologous to those of MMTV (Wang et al. 2004).

Mok et al. (2008) studied the integration of MMTV-like env sequences in human breast cancer. Sequence analysis identified a novel ORF of approximately 1.6 kb which is 94–99% identical to MMTV env genes. The MMTV-like env sequences were shown to be different from the human endogenous retroviral sequences and are closely related to rodents.

The involvement of MMTV in the pathogenesis of human breast cancer has long been assumed. However, this viral sequence has not been detected from many breast cancer samples in several subsequent studies as Japan, Germany, USA, Austria, Sweden and UK (Zangen et al. 2002; Witt et al. 2003; Mant and Cason 2004; Bindra et al. 2007; Frank et al. 2008; Fukuoka et al. 2008)

Table 3 shows studies of MMTV and breast cancer tissues using molecular biology involving methodology.

Conclusion

Breast cancer is the most frequently diagnosed malignancy of women in many populations. It is generally accepted that environmental factors play a role in the etiology of various types of cancer. However, viruses, such as specific types of human papillomavirus, Epstein–Barr and mouse mammary tumor virus, may be high-risk factors closely associated with human cancers. It is estimated that viruses are a contributory cause in 20% of all human cancers. The role of

Table 3 Results of studies into the presence of MMTV-like viruses genetic material in human breast cancer

Region	Sample	Controls/MMTV+ (%)	Cases	MMTV+ %	Authors
USA	Paraffin-embedded tissue/Frozen	107/(1.8)	314	38.5	Wang et al. (1995)
USA	Frozen	35/(0.0%)	73	37.0	Etkind et al. (2000)
Italy	Paraffin-embedded tissue	106/(0.9%)	106	30.1	Melana et al. (2001)
Argentina	Paraffin-embedded tissue	10/(10%)	74	31%	Melana et al. (2002)
USA	Paraffin-embedded tissue	–	18	0.0	Zangen et al. (2002)
Australia	Paraffin-embedded tissue	111/(1.8%)	45	42.2	Ford et al. (2003)
USA	Fresh frozen/Paraffin-embedded tissue	–	29	62	Wang et al. (2003)
Austria	Fresh frozen tumor	–	50	0.0	Witt et al. (2003)
Australia	Paraffin-embedded tissue	20/(0.0%)	33	78.8	Ford et al. (2004b)
Australia	Paraffin-embedded tissue	–	136	32	Ford et al. (2004a)
England	Fresh frozen tumor	–	44	0.0	Mant and Cason (2004)
Tunisia	Paraffin-embedded tissue	–	38	73.7	Levine et al. (2004)
USA	Paraffin-embedded tissue	–	12	50	Etkind et al. (2004)
Sweden	Fresh frozen	11/(0.0%)	18	0.0	Bindra et al. (2007)
Mexico	Paraffin-embedded tissue	–	119	4.2	Zapata-Benevides et al. (2007)
Tunisia	Frozen	–	122	13.9	Hachana et al. (2008)
USA	Paraffin-embedded tissue	–	3	100	Etkind et al. (2008)
Germany	Fresh Frozen	46/(0.0%)	23	0.0	Frank et al. (2008)
Japan		–	46	0.0	Fukuoka et al. (2008)

virus as a causative agent in human breast carcinogenesis has recently been the subject of renewed interest.

The identification of a mouse mammary tumor virus could support the viral etiology for breast tumors in animals, but similar viral sequences found in humans are not believed to play any direct role in carcinogenesis.

In virus-associated cancer, the tumor cells can exhibit viral antigens both internally or on their surfaces. As a result, viral antigens in tumors represent a potential antigenic target that is clearly different from normal tissues. This is potentially an exciting discovery; if a virus is found to present a definitive role in breast cancer development then the possibility of developing preventive treatments against such a virus becomes more plausible and may lead to a reduction in these cancers.

Recent technological advances now make it feasible to better tackle the methodological challenges of detecting virus in breast cancers. A critical next step in understanding this relationship is to apply detection strategies that are sensitive and specific for virus and able to localize this agent to particular malignant cells within the tissue. A recent National Cancer Institute recommendation specifies an approach combining real-time quantitative PCR, which allows measurement of the amount of viral load in archival tissue samples, with laser capture microdissection to improve localization of viral nucleic acid to benign or malignant components of a tissue sample.

In spite of many years of research, no etiological factors other than genetic susceptibility have been found for human breast cancer. If breast cancer has a viral etiology, it remains to be clarified.

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4 CONSIDERAÇÕES FINAIS

- Aumento da expressão relativa de RNAm de TLR3, IFNγ, CXCR4 e PKR foi verificado em cultura de PBMC humano na presença de RNA sintético dupla fita poli (I:C). Não foi observado efeito citotóxico e aumento na proliferação de células $CD3^+$, $CD4^+$ e $CD8^+$ nas culturas. Cultura de células sensibilizadas com RNA endógeno humano apresentou diminuição da expressão de RNAm de TLR3, IFNγ, CXCR4 e PKR quando comparada com cultura de células na ausência de estímulo.O RNA autólogo, endógeno, apresentou efeito inibitório sobre as células mononucleadas do sangue periférico humano. É possível que os RNAs endógenos como microRNAs e RNAs longos, não codificadores, através de suas complexas estruturas secundárias tenham envolvimento na regulação da expressão gênica independente do TLR3.
- A participação do RNA dupla fita e dos seus receptores na patogênese de doenças imunológicas e outras doenças, como câncer, podem ter implicações importantes na modulação da expressão gênica. Conhecimentos adquiridos a partir de estudos com RNAs endógenos como RNAs não codificadores ou reguladores podem contribuir para a compreensão de várias doenças onde os ácidos nucléicos podem desempenhar uma função importante na patogênese ou na regulação da expressão gênica.
- Aumento estatisticamente significante da expressão do TLR3 foi verificado no tecido mamário tumoral de pacientes sem acometimento de linfonodos. Correlação do aumento de expressão de RNAm para TLR3 e IFNγ, e TLR3 e CXCR4 foi verificado no tecido tumoral portanto é possível que estas moléculas tenham implicações na patogênese do câncer. É possível propor que a expressão aumentada de RNAm para IFNγ devido ao microambiente pró-inflamatório pode levar a um

aumento do mRNA para CXCR4 e, consequentemente, aumentar expressão de mRNA para TLR3 mesmo em pacientes sem acometimento de linfonodos.

APÊNDICE

APÊNDICE A

Estadiamento do Câncer de Mama Segundo Uicc

Para afirmar se um tumor está em estadiamento avançado ou não, usa-se um critério para a classificação dos tumores, criado pela União Internacional Contra o Câncer (UICC), denominado estadiamento, baseando-se no fato de que os tumores seguem um curso biológico comum. O estadiamento clínico é importante porque permite estabelecer a extensão e a gravidade da doença, planejar o tratamento, dar o prognóstico, ou seja, prever a evolução das enfermidades, e, finalmente, agrupar os casos para estudo e pesquisa (INCA/MS, 2007).

O Sistema Tumor-Nódulo-Metástase (TNM) foi desenvolvido por Pierre Denoix em meados de 1942 e representou uma tentativa de classificar o câncer baseado nos atributos morfológicos maiores dos tumores malignos que acreditavam influenciar o prognóstico da doença: tamanho do tumor primário (T), presença e extensão do envolvimento de nódulos linfáticos regionais (N), e presença de metástases distantes (M). A UICC apresentou a classificação clínica de câncer de mama baseada no Sistema TNM em 1958 e o Comitê Americano de Câncer (AJCC – American Joint Committee on Cancer) publicou um sistema de estadiamento de câncer de mama baseado no TNM no seu primeiro manual de estadiamento de câncer em 1977 (Beahrs, 1977). Desde então, revisões regulares têm sido emitidas para refletir maiores avanços em diagnósticos e tratamentos. Na revisão de 1987, diferenças entre as versões do AJCC e do UICC no sistema TNM foram eliminadas.

Portanto, esta avaliação tem como base a dimensão do tumor (T), a avaliação da extensão aos linfonodos (N) e a presença ou não de metástases à distância (M). Após a avaliação destes fatores, os casos são classificados em estádios que variam de I a IV graus crescentes de gravidade da doença (INCA/MS/2007).

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

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

TAMANHO DO TUMOR (T)

Tamanho do tumor (T)
• Tx - tumor não pode ser avaliado

- T0 - não há evidência de tumor primário
- Tis - carcinoma *in situ*
- T1 - tumor com até 2 cm em sua maior dimensão
- T1 mic - carcinoma microinvasor (até 1 mm)
- T1a - tumor com até 0,5 cm em sua maior dimensão
- T1b - tumor com mais de 0,5 e até 1 cm em sua maior dimensão
- T1c - tumor com mais de 1 cm e até 2 cm em sua maior dimensão
- T2 - tumor com mais de 2 e até 5 cm em sua maior dimensão
- T3 - tumor com mais de 5 cm em sua maior dimensão
- T4 - qualquer T com extensão para pele ou parede torácica
- T4a - extensão para a parede torácica
- T4b - edema (incluindo *peau d'orange*), ulceração da pele da mama, nódulos cutâneos satélites na mesma mama
- T4c - associação do T4a e T4b
- T4d - carcinoma inflamatório

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

LINFONODOS REGIONAIS (N)

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

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

METÁSTASES (M)

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

ESTADIAMENTO TNM DO CÂNCER DE MAMA POR AGRUPAMENTOS

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

* qq = qualquer

Referências:

Beahrs OH.; CARR DT; RUBIN P. Manual for Staging of Cancer. Philadelphia: Lippincott, 1977.

INCA/MS: Instituto Nacional de Câncer do Ministério da Saúde. 2007 Disponínel em: < 13 http://www.inca.gov.br/estimativa/2008/versaofinal.pdf > Acesso em Dezembro 2010.

APÊNDICE B

Aprovação no Comitê de Ética em Pesquisa em Seres Humanos Universidade Estadual de Londrina

"Análise da região 3UTR da quimiocina SDF-1, expressão do receptor CXCR4 e quimiocinas: implicações na patogênese do câncer de mama"

CAAE - 0278.0.268.000-06

Título do Projeto de Pesquisa
Análise da região 3UTR da quimiocina SDF-1, expressão do receptor CXCR4 e quimiocinas: implicações na patogênese do câncer de mama

Situação	Data Inicial no CEP	Data Final no CEP	Data Inicial na CONEP	Data Final na CONEP
Aprovado no CEP	11/12/2006 10:41:28	06/02/2007 18:54:23		

Descrição	Data	Documento	Nº do Doc	Origem
2 - Recebimento de Protocolo pelo CEP (Check-List)	11/12/2006 10:41:28	Folha de Rosto	0278.0.268.000-06	CEP
1 - Envio da Folha de Rosto pela Internet	05/12/2006 17:35:21	Folha de Rosto	FR118144	Pesquisador
3 - **Protocolo Aprovado no CEP**	06/02/2007 18:54:23	Folha de Rosto	322/06	CEP

APÊNDICE C

Aprovação no Comitê de Ética em Pesquisa em Seres Humanos Universidade Estadual de Londrina

"Análise da expressão de genes relacionados a células T reguladoras (Tregs) FoxP3+ em Pacientes com câncer de mama"

CAAE - 0179.0.268.000-09

Título do Projeto de Pesquisa
Análise da expressão de genes relacionados a células T reguladoras (Tregs) FoxP3+ em Pacientes com câncer de mama

Situação	Data Inicial no CEP	Data Final no CEP	Data Inicial na CONEP	Data Final na CONEP
Recebido no CEP	10/10/2009 15:03:01			

Descrição	Data	Documento	Nº do Doc	Origem
2 - Recebimento de Protocolo pelo CEP (Check-List)	10/10/2009 15:03:01	Folha de Rosto	0179.0.268.000-09	CEP
1 - **Protocolo Aprovado no CEP**	29/09/2009 22:55:58	Folha de Rosto	FR294246	Pesquisador

APÊNDICE D

Aprovação no Comitê de Ética em Pesquisa em Seres Humanos Universidade Estadual de Londrina

"Análise do polimorfismo da quimiocina SDF-1 humana e a expressão de RNAm do seu receptor CXCR4 em pacientes com câncer"

CAAE - 0005.0.268.000-06

Título do Projeto de Pesquisa
Análise do polimorfismo da quimiocina SDF-1 humana e a expressão de RNAm do seu receptor CXCR4 em pacientes com câncer

Situação	Data Inicial no CEP	Data Final no CEP	Data Inicial na CONEP	Data Final na CONEP
Aprovado no CEP	21/02/2006 15:04:10	16/05/2006 09:54:23		

Descrição	Data	Documento	Nº do Doc	Origem
2 - Recebimento de Protocolo pelo CEP (Check-List)	21/02/2006 15:04:10	Folha de Rosto	0005.0.268.000-06	CEP
1 - Envio da Folha de Rosto pela Internet	12/02/2006 22:41:19	Folha de Rosto	FR84253	Pesquisador
3 - **Protocolo Aprovado no CEP**	16/05/2006 09:54:23	Folha de Rosto	052/06	CEP