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VASCO JOSÉ RAMOS MALTA PATU

AVALIAÇÃO IMUNOQUIMILUMINESCENTE EM LESÕES TUMORAIS DE MAMA

Recife, 2012

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Tese de Doutorado apresentada ao Programa de Pós-Graduação em Ciências Biológicas, do Centro de Ciências Biológicas da Universidade Federal de Pernambuco, como requisito para a obtenção do título de Doutor em Ciências Biológicas. Área de Concentração: Biotecnologia.

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DEDICATÓRIA

A minha esposa, a meus pais, a minha irmã e aos meus sogros que apoiaram minhas decisões, e me deram todo o carinho durante mais uma jornada.

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RESUMO

Embora o estudo imunoistoquímico seja um método bastante utilizado na rotina do diagnóstico anatomopatológico, este teste ainda apresenta importantes limitações principalmente quanto a incapacidade de distinção entre alguns tipos de lesões tumorais. Dessa maneira, outras ferramentas vêm sendo desenvolvidas a fim de aumentar a sensibilidade e especificidade da identificação antígeno-anticorpo. Dentre estas, os ensaios quimiluminescentes vem sendo cada vez mais utilizados em estudos para o mapeamento de amostras biológicas para fins diagnósticos. Dentre os inúmeros benefícios da utilização dos métodos quimiluminescentes, podemos citar o limite de detecção ultra-sensível, testes rápidos e um amplo campo de aplicações. O presente estudo buscou elaborar um protocolo para detecção da proteína galectina-3 em tecidos tumorais da mama a partir de anticorpo conjugado ao éster de acridina realizando ensaios imunoquimiluminescentes e compará-los aos resultados da imunohistoquímica convencional. O anticorpo monoclonal anti-galectina-3 foi conjugado ao éster de acridina e esse conjugado foi submetido à ensaios imunoquimiluminescentes em tecidos de mama além da imunohistoquímica convencional. Nossos resultados demonstram a eficiência na conjugação da anti-galectina-3 ao éster de acridina, um *shelf-life* do conjugado anticorpo/éster de acridina (AC-EA) com aproveitamento de 96,51% após 12 meses e a aplicação desse conjugado em ensaios imunoquimiluminescentes nos tecidos mamários com carcinoma ductal infiltrante (39.8 x 104 ± 29344) e com fibroadenoma (88.88 x 104 ± 17880) demonstraram padrões diferenciais entre a sua contraparte normal (58.87 x 104 ± 17880). Concluiu-se então, que o ensaio imunoquimiluminescente proposto pode ser utilizado como método diagnóstico alternativo na identificação de antígenos principalmente em amostras teciduais pequenas.

Palavras-chave: imunoquimiluminescência, tumores, éster de acridina, galectina-3, mama.

ABSTRACT

Although immunohistochemistry is a method widely used in routine pathological diagnosis, this test also shows significant limitations especially regarding the inability to distinguish between some types of tumors. Thus, other tools have been developed to increase the sensitivity and specificity of antigen-antibody identification. Among these methods, chemiluminescent assays are being increasingly used in studies for the mapping of biological samples for diagnostic purposes. There are numerous benefits related to chemiluminescent methods, like a limit of ultra-sensitive detection, rapid testing and a wide range of applications. This study aims to develop a protocol for detection of protein galectin-3 in tumor tissues of the breast using antibody conjugated to acridinium ester (AB-AE) for chemiluminescent immunoassay tests and compared them with results of conventional immunohistochemistry. The monoclonal anti-galectin-3 was conjugated to acridinium ester and this conjugate was subjected to chemiluminescent immunoassay in tissues of breast in addition to conventional immunohistochemistry. Our results demonstrate the efficiency of conjugation of anti-galectin-3 to the acridine ester, a shelf-life of the conjugate AB-AE with utilization of 96.51% after 12 months and the application of combined AB-AE tests in breast tissues. Chemiluminescent immunoassay showed different patterns between the studied tumoral lesions (invasive ductal carcinoma- 39.8 x 104 ± 29344 and fibroadenoma- 88.88 x 104 ± 17880) and their normal counterpart (58.87 x 104 ± 17880). It was concluded that the proposed chemiluminescent immunoassay test can be used as an alternative diagnostic method for identifying antigens mainly in small tissue samples.

Keywords: chemiluminescent immunoassay, tumors, acridinium ester, galectin-3, breast.

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Capítulo I

LISTA DE SIGLAS E ABREVIATURAS

Bcl-2 - B-cell lymphoma 2
CRD – Carbohydrate Recognition Domais
DAB - Diaminobenzidina
EA - Éster de Acridina
HE - hematoxilina e eosina
IARC – International Agency for Research of Cancer
IgG - Immunoglobulin G
INCA - Instituto Nacional do Câncer
kDa - Kilodalton
OMS - Organização Mundial da Saúde
PBS - Phosphate Buffered Saline
PCNA – Proliferating Cell Nuclear Antigen
RLU - Relative Light Unit

SUMÁRIO

1. INTRODUÇÃO

O câncer de mama é um dos tipos mais freqüentes de neoplasias entre mulheres, contribuindo com pouco mais de um milhão de casos novos ao ano em todo o mundo. No Brasil, é o que mais causa mortes entre as mulheres, sendo que em 2000, foram registradas 8.390 mortes decorrentes desse tipo de câncer. O Ministério da Saúde, pelo Programa Nacional de Controle do Câncer, estima que dos 489.270 novos casos de câncer que foram diagnosticados em 2010, o câncer de mama foi o segundo mais incidente entre a população feminina e responsável por 49.240 casos (INCA, 2010).

Associado a fatores genéticos, estudos epidemiológicos conduzidos em diferentes populações determinaram que fatores de risco como idade, localização geográfica, status socioeconômico e eventos reprodutivos (idade da menarca e da menopausa, infertilidade, gravidez e amamentação) estão associados à maior incidência de carcinoma de mama (Dumitresco & Cotarla, 2005).

As galectinas vêm sendo largamente utilizadas como ferramentas imunohistoquímicas para descrever alterações na superfície das células tumorais onde essas alterações são associadas ao crescimento de células tumorais na indução de apoptose ou metástase (Araújo-Filho *et al*., 2006; Elola *et al.*, 2005; Dumic, 2006).

Existem atualmente diversas técnicas diagnósticas que auxiliam no tratamento das neoplasias, dentre elas podemos destacar a imunohistoquímica de biópsias teciduais, método este de grande auxilio na discriminação de tumores assemelhados e, muitas vezes, de difícil interpretação pelos métodos histológicos de rotina (Novellino, et al., 2003; Paveli, et al., 2006). Além disso, os métodos imunohistoquímicos quando analisados de forma qualitativa, muitas vezes têm apresentado grande disparidade e variabilidade de resultados entre diferentes observadores. Assim, a necessidade de uma escala numérica e reprodutível dos padrões de marcação dos tecidos, aumentando a sensibilidade e o controle de qualidade das análises, tem sido buscada através de refinamentos tecnológicos empregando métodos quantitativos automatizados (Rashbass *et al.*, 2000; Novik *et al.*, 2000; Ram & Siar, 2005).

Análises laboratoriais baseadas nos princípios quimiluminescentes (QL) têm sido desenvolvidas para dosagens que requerem uma alta sensibilidade, como a determinação de citocinas, fatores de crescimento e proteínas relacionadas a processos patológicos específicos. Dentre a grande variedade de testes já disponíveis comercialmente estão

aqueles utilizados na avaliação de função tireoidiana, fertilidade, marcadores tumorais, monitoramento de drogas terapêuticas, hepatite, proteínas específicas e esteróides (Kricka, 2003).

Substâncias quimiluminescentes podem ser detectadas na faixa de fentomoles ou atomoles (10^{-15} a 10^{-18} mol), com sensibilidade superior aos ensaios espectofotométricos (10^{-6} a 10^{-9} mol) e fluorimétricos (10^{-9} a 10^{-12} mol) (Campbell *et al.,* 1985). Vários compostos orgânicos exibem QL em condições apropriadas, dentre os quais se destacam o luminol, isoluminol, éster de acridina ou seus derivados como alguns dos marcadores utilizados em imunoensaios quimiluminescentes (Roda *et al.,* 2000).

O éster de acridina foi introduzido em imunoensaios a partir da necessidade de substituir os marcadores radioisotópicos que estavam se tornando poucos populares devido às suas curtas meia vidas, ao perigo potencial à saúde e aos problemas quanto aos dejetos gerados (Weeks *et al.*, 1983). Desde então o éster de acridina tem sido foco de pesquisas para ampliar suas aplicações nas mais diversas áreas de Biotecnologia (Adamczyk *et al.,* 2001).

Recentemente, demonstramos que a lectina marcada com éster de acridina poderia ser utilizada em histoquímica (Campos et al., 2006) em substituição à marcação com peroxidase. Apesar de todas as interrelações feitas entre ensaios quimioluminescentes com lectinas e imunoquimiluminescentes com anticorpos (Dreveny et al., 1999; Yang et al., 2002) não foram encontrados trabalhos demonstrando a aplicabilidade do emprego desta técnica na marcação de lesões tumorais benignas ou malignas de mama.

2. REVISÃO DA LITERATURA

2.1 Câncer, uma visão geral

A carcinogênese inicia-se a partir de uma alteração no DNA celular ou da ativação anormal de genes presentes em células sadias. Tais genes coordenam a proliferação, a diferenciação, à morte por apoptose e outras funções celulares primordiais. A expressão amplificada de proteínas reguladas por tais genes pode determinar a progressão de alterações celulares levando aos estados neoplásicos malignos (Hanson & Hodgson, 2010).

Segundo relatório da Agência Internacional para Pesquisa em Câncer (IARC)/OMS, o impacto global do câncer dobrou em 30 anos. Estimou-se que, no ano de 2008, ocorreriam cerca de 12 milhões de novos casos de câncer e 7 milhões de óbitos, e que 84 milhões é o número de vítimas fatais do câncer no período de 2005 à 2015 (World Cancer Report, 2008).

No Brasil, as estimativas para o ano de 2010/2011 apontam que ocorrerão 489.270 casos novos de câncer (INCA - Instituto Nacional do Câncer, 2010) (**Figura 1**).

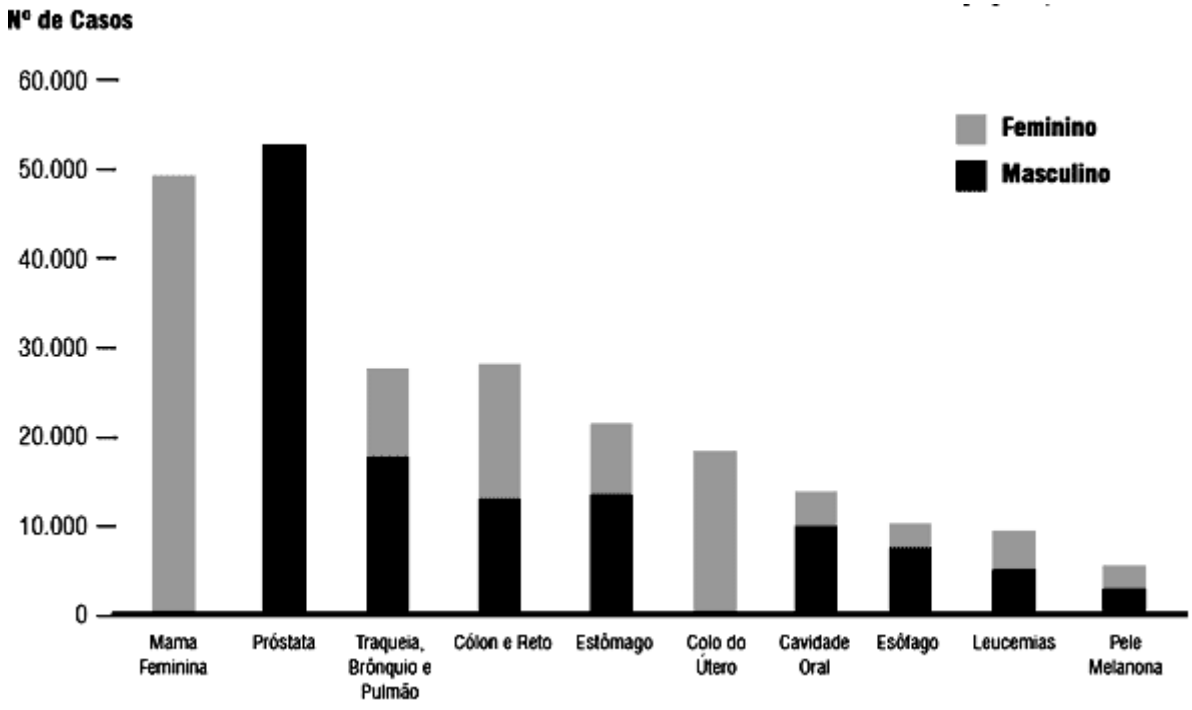

Figura 1: Estimativas de cancer para o ano de 2010/2011, exceto pele não melanoma. Fonte: INCA, 2010.

No que se refere às funções bioquímicas, as moléculas de adesão e vias de transdução dos sinais para o crescimento tumoral são bastante alterados durante a progressão tumoral. As proteínas normais codificadas pelos proto-oncogenes (genes que regulam a proliferação celular) desempenham funções iniciais nas células durante o desenvolvimento das neoplasias. Dentre estas moléculas, destacam-se os fatores de crescimento, proteínas envolvidas na recepção e transdução de sinais localizados na superfície celular (Gua *et al*. 2006).

Pelo menos no último século, todos os esforços intelectuais e tecnológicos empreendidos na cura dessa doença, contribuíram para revelar uma complexa série de mecanismos de comunicação molecular, intra e intercelular que, em condições fisiológicas, atua na manutenção dos sistemas vivos. Essa complexa rede de sinalização é constituída de vias metabólicas de manutenção e transmissão da informação genética e da síntese e regulação de macromoléculas, como as proteínas, contendo inúmeros pontos de regulação, sinais divergentes e locais de cruzamento com outras vias e cascatas de sinalização, exercendo mútuas influências (Taniguchi *et al.,* 2006).

As proteínas normais codificadas pelos proto-oncogenes (genes que regulam a proliferação, divisão e diferenciação celular) desempenham funções iniciais nas células durante o desenvolvimento das neoplasias. Dentre estas moléculas, destacam-se os fatores de crescimento, proteínas envolvidas na recepção e tradução de sinais localizados na superfície celular (Goo *et al.,* 2010).

O mecanismo de reconhecimento molecular na superfície da célula é afetado quando células normais sofrem o processo de neoplasia. O resultado é um crescimento e divisões descontroladas, devido às alterações nos mecanismos de reconhecimento que agem na membrana celular (Shekhar *et al.,* 2004).

Nesse contexto o estudo histoquímico do tecido obtido a partir de uma amostra do tumor ou da peça completa dos tumores assume grande importância prática no momento de decidir sobre o tratamento de cada paciente. Embora um câncer possa ser fortemente sugerido clínica e radiologicamente, seu diagnóstico não pode ser definido até que se obtenha confirmação citológica ou histológica da lesão. Sem a prova anatomopatológica da malignidade, o tratamento não pode ser adequadamente planejado, procedimentos terapêuticos não podem ser comparados, nem qualquer prognóstico pode ser feito de maneira precisa e eficiente (Faria, 1999).

Estudos com extratos de tumores realizados na década de 30 foram os pioneiros em demonstrar alterações bioquímicas nas células tumorais, o que foi posteriormente confirmado por estudos que demonstraram padrões aberrantes de glicosilação associados ao tumor (Dabelsteen *et al.,* 1992, Melo-Junior *et al.,* 2008).

Transformações celulares são freqüentemente acompanhadas por um aumento geral no tamanho dos glicolipídeos do metabolismo. Com o advento da tecnologia de anticorpos monoclonais, descobriu-se que muitos dos anticorpos "tumor-específicos" reconhecem os epítopos carboidratados especialmente em glico-esfingolipídeos. Além do que, correlações significativas entre certos tipos de glicosilação alterada e o atual prognóstico de tumores referidos de animais experimentais ou humanos aumentam o interesse sobre essas mudanças bioquímicas específicas (Veiga *et al.,* 2009).

2.2 Lesões tumorais da mama

As glândulas mamárias ficam sobre o músculo peitoral na parede torácica superior. São compostas por tecido especializado glandular suportado por tecido conectivo predominantemente adiposo, com agrupamentos neurovasculares e uma quantidade variável de tecido fibroso (Cunha *et al*., 2004).

Seis a dez dos principais sistemas ductais originam-se no mamilo. O epitélio escamoso queratinizado da pele sobrejacente continua dentro dos ductos e então muda abruptamente para um epitélio cubóide de camada dupla. Um pequeno tampão de queratina é encontrado, com freqüência, no orifício do ducto. A pele alreolar circunjacente é pigmentada e sustentada por musculatura lisa (Robbins *et al*., 2010).

A ramificação sucessiva de grandes ductos leva, eventualmente, à unidade ductolobular terminal. Na mulher adulta, as ramificações do ducto terminal criam um agrupamento de ácinos que formam um lóbulo. Cada sistema ductal ocupa tipicamente um quarto da mama, e sobrepõem-se extensivamente sobre outros. Em algumas mulheres, os ductos estendem-se ao tecido subcutâneo da parede torácica e da axila (Ian & D'Arrigo, 2004).

Na mama normal (**Figura 2**) os ductos e lóbulos são forrados por dois tipos de células. Uma camada inferior plana e descontínua de células contráteis contendo miofilamentos (células mioepiteliais) que ficam sobre a membrana basal. Essas células ajudam na ejeção do leite durante a lactação e têm um papel importante na manutenção da

estrutura e função normais do lóbulo e da membrana basal. Uma segunda camada de células epiteliais demarca os lúmens. As células luminais do ducto terminal e do lóbulo produzem leite, porém aquelas que revestem o grande sistema ductal não (Ian & D'Arrigo, 2004).

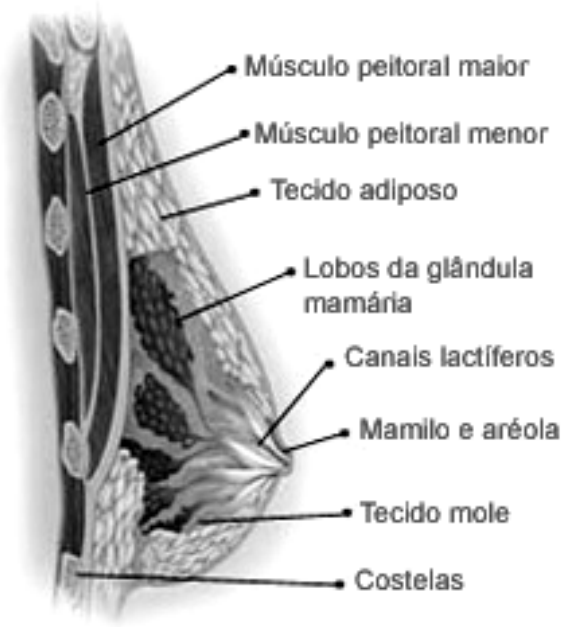

Figura 2**.** Aspecto anatomo-funcional de mama normal de mulher adulta. Fonte: http://www.mentorcorp.com/global

A maioria do estroma mamário consiste em tecido conjuntivo fibroso denso misturado com tecido adiposo (estroma interlobular). Os lóbulos são confinados por um estroma mamário específico hormonalmente responsivo, delicado e mixomatoso que contém uma disseminação de linfócitos, chamado estroma intralobular (Robbins *et al.,* 2010).

As células que apresentam algum tipo de lesão tumoral carregam uma capacidade proliferativa indefinida, sendo capazes de mascarar o compromisso de diferenciação terminal e a quietude pós-mitótica que regulam normalmente a homeostase do tecido em um organismo. Essa proliferação descontrolada das células é também, freqüentemente, conseguida por uma desregulação direta do controle do ciclo celular (Herzig e Christofori, 2002; Duffy *et al.,* 2010).

A Organização Mundial da Saúde estima que, por ano, ocorram mais de 1.29 milhões de casos de câncer de mama em todo o mundo, o que o torna o câncer mais comum entre as mulheres (WHO, 2008).

Tem-se observado, em alguns países desenvolvidos, como é o caso dos Estados Unidos, Canadá, Reino Unido, Holanda, Dinamarca e Noruega, um aumento da incidência do câncer de mama acompanhado de uma redução da mortalidade por esse câncer, o que está associado à detecção precoce por meio da introdução da mamografia para rastreamento e à oferta de tratamento adequado. Em outros países, como no caso do Brasil, o aumento da incidência tem sido acompanhado de um agravo da mortalidade, o que pode ser atribuído, principalmente, a o diagnóstico tardio e a instituição de terapêutica adequada (INCA, 2010).

A vasta exibição de semelhanças histológicas de doença mamária proliferativa atípica, bem como carcinomas, traduz-se nas manifestações externas de centenas de alterações biológicas. Atualmente, nenhuma alteração genética ou funcional comum pode ser encontrada em cada tipo de câncer de mama. A maioria das alterações reportadas ocorre em somente um subconjunto de carcinomas (Mirtrunen & Hirvonen, 2003).

As alterações morfológicas na mama associadas ao menor risco de câncer são lesões com números crescentes de células epiteliais (hiperpalsias mamárias). Isto sugere que as alterações iniciais estão relacionadas à evasão de sinais inibidores do crescimento, da apoptose e da auto-suficiência dos sinais de crescimento. Há evidências de que, mesmo no estágio inicial, exista expressão anormal dos receptores hormonais e regulação anormal da proliferação em associação à positividade dos receptores hormonais (Iqbal *et al*., 2001).

O fibradenoma é o tumor benigno mais freqüente em mulheres 20 e 30 anos de idade. Embora classificado entre as neoplasias benignas, alguns autores o consideram uma lesão hiperplásica. Como regra geral, o fibroadenoma apresenta-se como nódulo único, bem delimitado, em pacientes jovens. Durante a gravidez e no final do ciclo menstrual, o fibroadenoma pode sofrer aumento de volume, macroscopicamente, o tumor é bem circunscrito, têm consistência elástica e em geral mede de 1 a 3 centímetros. Os tumores são nódulos branco-acinzentados, firmes que se destacam acima do tecido circunjacente e contêm com freqüência, espaços do tipo fendas (**figura 3**). Na intimidade do fibroadenoma, podem ser encontradas várias das alterações que ocorrem nos lóbulos, como adenose esclerosante, metaplasia apócrina, hiperplasias epiteliais e raramente

carcinoma in situ ou invasores. Já no estroma se identificam importantes alterações no tecido conjuntivo de sustentação podendo ocorrer fibrose e depósitos hialinos (Ooe *et al*., 2011).

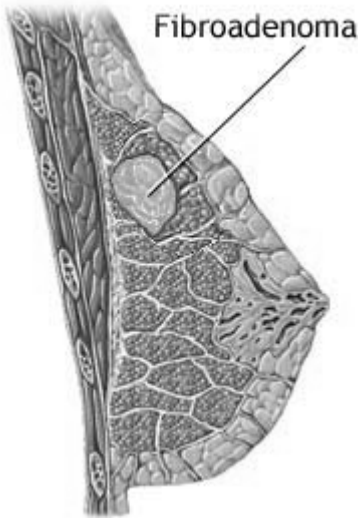

Figura 3**.** Aspecto morfológico geral de fibroadenoma. Fonte: http://www.insightec.com

O estágio final da carcinogênese, a transição do carcinoma limitado pela membrana basal dos ductos e lóbulos (carcinoma *in situ*) ao carcinoma infiltrante, é o menos compreendido. As funções gênicas específicas necessárias à invasão são de difícil identificação (Porter *et al,* 2001). O desequilíbrio cromossomal ocorre com os ganhos ou perdas em múltiplos *locus*, como em lesões hiperplásicas que progridem para carcinoma ductal *in situ* até carcinoma ductal infiltrante. Não obstante, as mudanças cromossômicas compartilhadas com ambos os carcinomas ductal *in situ* e adjacente ao carcinoma ductal infiltrante demonstram sua relação clonal e evolucionária (Menezes *et al.,* 2006).

Dentre os tumores malignos de mama, o carcinoma ductal infiltrante representa o maior grupo, constituindo cerca de 65 a 80% dos carcinomas mamários (Dantas *et al*., 2003). O carcinoma ductal infiltrante pode ser de origem familiar ou esporádica, estima-se que entre 5 e 10% dos casos são de origem familiar. As aberrações cromossômicas encontradas não são específicas para este tumor e os cariótipos indicam um alto grau de heterogeneidade nestes tumores (Carabias-meseguer, 2011).

2.3 Galectinas, definições e aplicações

As galectinas são proteínas da família das lectinas animais (lectinas endógenas), envolvidas em diversos processos biológicos, tais como adesão intercelular, controle do ciclo celular, proliferação celular, resposta imune, apoptose e em processos neoplásicos. (Liu *et al.,* 2002).

Até o momento foram descritas 15 galectinas oriundas de mamíferos e essa família de proteínas é subdividida em 3 categorias com base na homologia das seqüências de aminoácidos: I) "prototypical", são aquelas que contem um único CRD (carbohydrate recognition domains) e formam homodímeros não-covalentes; II) "Chimeric", apresentam um CRD e um domínio amino-terminal rico em resíduos de prolina, glicina e tirosina; o qual é sensível a metaloproteinases e contribui para a oligomerização dessas lectinas; III) "Tandem-Repeat", polipeptídeos únicos compostos por dois CRDs distintos conectados por um peptídeo ligador de 5 a 50 resíduos de aminoácidos (Cummings & Liu, 2009).

A estrutura molecular das proteínas dessa família geralmente consiste de cerca de 130 aminoácidos, altamente conservados, bem como uma região de reconhecimento a carboidratos por reconhecer estruturas de β-galactosídeos (Barondes *et al.,* 1994; Lahm *et al.,* 2004) (Figura 4).

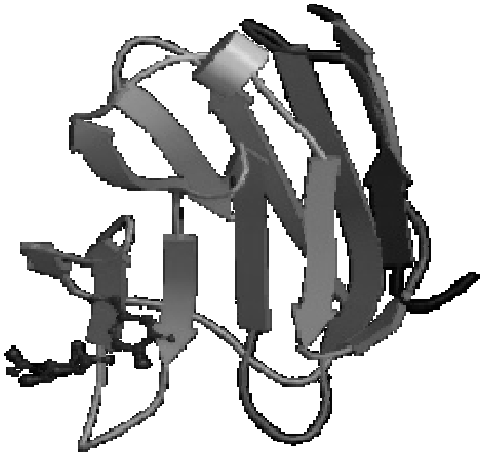

Figura 4. Estrutura molecular da galectina-3 complexada ao carboidrato.
(fonte: http://web.mit.edu/glycomics/moleculepages/cbp/galectins/gal3_human/)

Dentre essas proteínas identificadas em mamíferos, a galectina-3, é uma das mais estudadas e é monomérica de 30 Kda indicada como um potencial marcador tumoral de malignidade nas neoplasias (Califice *et al.,* 2004). Ela faz parte das lectinas com afinidade por beta-galactosideo, sua expressão vem sendo relacionada ao surgimento de tumores e metástases (Christoph *et al.,* 2004; Alves *et al.,* 2010).

Sua estrutura parece ser única quando comparada com as demais galectinas de vertebrados (Houzelstein *et al.,* 2004). Ela foi inicialmente identificada como marcador de diferenciação de macrófagos (Ho *et al.,* 1982).

Na maioria das vezes são encontradas no citoplasma celular, algumas são secretadas pelas células e interagem com proteínas da superfície celular regulando a adesão entre as células e destas entre a matriz extracelular (Hughes, 2001; Martinez *et al.,* 2004).

Essas proteínas vêm sendo largamente utilizadas como ferramenta na imunohistoquímica. (Rabnovich *et al.,* 2002; Elola *et al.,* 2005).

Uma série de evidências experimentais e clinicas vêm reforçado as correlações entre sua expressão e as neoplasias. Estudos demonstram que a presença de galectinas é relevante na progressão de neoplasias em certos tumores malignos, indicando estas como potenciais marcadores tumorais (Pacis *et al.,* 2000; Califice *et al.,* 2004).

Ela é encontrada em diversos tecidos normais, e sua expressão em adultos é similar à expressão durante a embriogênese, isso foi relatado para células epiteliais e mielóides. A Galectina-3 foi encontrada nas células do epitélio intestinal (Tabela 1).

Tabela 1. Tecidos e células humanas que expressam galectina-3.

Local	Referência
Células do epitélio intestinal	Lotz *et al.,* 1993
Epitélio do cólon	Lotz *et al.,* 1993
Epitélio córneo	Gupta *et al.,* 1997; Cao *et al.,* 2002
Epitélio conjuntivo	Hrdlickova *et al.,* 2001
Epitélio olfatório	Heilmann *et al.,* 2000
Células epiteliais do rim	Sasaki *et al.,* 1999
Pulmão	Kasper *et al.,* 1996
Timo	Villa-Verde *et al.,* 2002
Mama	**Castronovo *et al.,* 1996**
Tireóide	Saggiorato *et al.,* 2004
Próstata	Pacis *et al.,* 2000
Células ductais das glândulas salivarias	Xu *et al.,* 2000
Pâncreas	Schaffert *et al.,* 1998
Rim	Sasaki *et al.,* 1999
Olho	Fautsch *et al.,* 2003
Ductos biliares	Shimonishi *et al.,* 2001
Fibroblastos	Moutsatsos *et al.,* 1987
Condrócitos	Colnot *et al.,* 1999
Osteoblastos	Stock *et al.,* 2003
Osteoclastos	Niida *et al.,* 1994
Ceracinócitos	Wollenberg, 1993; Konstantinov, 1994
Células de Schwann	Reichert *et al.,* 1994
Mucosa gástrica	Lotan *et al.,* 1994a
Células endoteliais	Lotan *et al.,* 1994b
Neutrófilos	Truong *et al.,* 1993a
Eosinófilos	Truong *et al.,* 1993b
Basófilos	Frigeri *et al.,* 1993
Mastócitos	Craig *et al.,* 1995
Células de Langerhans	Wollenberg 1993; Smetana *et al.,* 1999
Células denditricas	Flotte *et al.,* 1983; Dietz *et al.,* 2000
Monócitos	Li *et al.,* 1995
Macrófagos	Flotte *et al.,* 1983; Kasper *et al.,* 1996; Saada *et al.,* 1996; Maeda *et al.,* 2003

Alterações no perfil de expressão da galectina-3 vêm sendo observado na progressão maligna de diversos tipos de câncer como no de cólon, tireóide, mama, estômago (Miyazaki *et al.,* 2002) (Tabela 2).

Tabela 2. Padrão de marcação através de imunohistoquímica para galectina-3 em lesões tumorais:

Tecido/Órgão	Perfil de expressão	Referência
Estômago	Aumentado	Miyazaki *et al.,* 2002
Linfonodos	Aumentado	Miyazaki *et al.,* 2002
Cabeça e Pescoço	Aumentado	Gillenwater *et al.,* 1996
Língua	Aumentado	Honjo *et al.,* 2000
Hepatocelular	Aumentado	Hsu *et al.,* 1999; Chung *et al.,* 2002
Cólon	Aumentado	Irimura *et al.,* 1991; Lotan *et al.,* 1991
Tireóide	Aumentado	Bartolazi *et al.,* 2008, Inohara *et al.,* 2008
Glândulas salivares	Reduzido	Xu *et al.,* 2000
Mama	**Reduzido**	**Stannard *et al.*, 2010**
Ovário	Reduzido	Van den Brule *et al.,* 1994
Próstata	Reduzido	Araújo-Filho *et al.,* 2002 Melo-Junior *et al.,* 2010
Útero	Reduzido	Van den Brule *et al.,* 1996
Células basais de pele	Reduzido	Castronovo *et al.,* 1991
Pele	Reduzido	Mollenhauer *et al.,* 2003
Metástases do fígado	Reduzido	Inufusa *et al.,* 2001
Pâncreas	Reducido	Schaffert *et al.,* 1998
Adenocarcinoma	Reduzido	Inufusa *et al.,* 2001

Após sintetizada, localiza-se predominantemente no citoplasma podendo ser encontrada também no núcleo, sugerindo que esta proteína fica alternando entre núcleo e citoplasma (Davidson *et al.,* 2002).

Alguns trabalhos sugerem que a galectina-3, quando localizada no compartimento extracelular exerce uma função anti-apoptótica. Pode não ser uma molécula anti-apoptótica por si só, mas existe a possibilidade de que a sua interação com alguma proteína na superfície celular possa exercer essa atividade em algumas células, contribuindo para a sobrevivência celular (Nakahara *et al.,* 2005).

O mecanismo molecular de regulação da apoptose ainda não está bem esclarecido, contudo, é possível que ela possa mimetizar a Bcl-2, uma proteína mitocondrial bem conhecida como supressora da apoptose (Yang *et al.,* 1996; Moon *et al.,* 2001). Apesar de existirem inúmeros trabalhos na literatura relacionando a expressão da galectina-3 à invasão tumoral e metástases em diversas neoplasias, alguns dados são conflitantes e

inconsistentes e não há um consenso geral na literatura a respeito do papel dessa proteína na progressão tumoral (Dumic *et al.,* 2006).

Por outro lado, em algumas lesões como tumores de mama, próstata e ovário, a expressão da galectina-3 é inversamente proporcional ao potencial metastático da lesão (Castronovo *et al.,* 1996; Lipinska, 2001).

Alguns trabalhos com cultura de células mostraram que a diminuição da expressão dessa proteína está diretamente envolvida na progressão de tumores de mama. (Young et al., 2002).

2.4 Aplicações da quimiluminescência na saúde

A quimiluminescência (QL) é definida como o processo no qual moléculas ou átomos excitados através de reações químicas liberam o excesso de energia na forma de luz. Este processo envolve a absorção, pelos reagentes, de energia suficiente para a geração de um novo complexo, eletronicamente excitado. Se a espécie excitada for emissiva, esta produzirá a radiação diretamente, caso contrário, pode ocorrer transferência de energia do estado excitado formado por uma molécula aceptora apropriada, resultando na emissão indireta da radiação (Albertin *et al.,* 1998; Yang *et al*., 2008).

Dentre os inúmeros benefícios da utilização dos métodos luminescentes podemos citar: limite de detecção ultra-sensível, testes rápidos e um amplo campo de aplicações analíticas, toxicidade inferior comparada à oferecida por radioisótopos, não agressão ao meio ambiente e especificidade (Ferreira & Rossi, 2002). A quimioluminescência tem também como grande vantagem ser um sistema frio uma vez que a energia na forma de luz é produzida diretamente de uma reação química, sem passar por um estágio intermediário envolvendo calor (Catalani, 1996).

A aplicação da QL como instrumento analítico depende da acoplagem da substância de interesse a um dos elementos participantes da reação quimiluminescente ou ao produto no estado excitado. A concentração da amostra desconhecida será proporcional à produção de luz total emitida ou a um parâmetro físico associado à luminescência, tal como cor ou polarização da luz emitida (Campbell *et al.,* 1985).

Algumas análises laboratoriais baseadas em princípios quimiluminescentes têm sido desenvolvidas para dosagens que requerem uma alta sensibilidade, como a determinação de citocinas, fator de crescimento epidérmico, e de crescimento endotelial

vascular. Dentre a grande variedade de testes já disponíveis comercialmente estão aqueles utilizados na avaliação de função tireoidiana, dosagem de PSA, fertilidade, marcadores tumorais, monitoramento de drogas terapêuticas, hepatite, proteínas específicas e esteróides (Kricka 2003).

Devido à necessidade de substituir os marcadores radioisotópicos que estavam se tornando poucos populares devido à sua curta meia vida, ao perigo potencial à saúde e aos problemas quanto aos dejetos gerados, o éster de acridina foi introduzido em imunoensaios (Weeks *et al.,* 1983). Desde então o éster de acridina tem sido foco de pesquisas para ampliar sua aplicação nas mais diversas áreas de Biotecnologia. (Adamezyk *et al.,* 2001).

A acridina é um composto fluorescente cujos sais podem ser estimulados para produzir luz na presença de peróxido de hidrogênio em meio alcalino diluído (**Figura 5**) na ausência de um catalizador (Weeks *et al*., 1983). Essa propriedade se deve a presença de um nitrogênio quaternário central e a derivatização no carbono 9 que possui um Ester de fenil marcado com um radical. Dentre os derivados de acridina, o ester de acridina recebeu um destaque especial pela fácil oxidação com o peróxido de hidrogênio e a formação de um produto excitado (N-metilacridona), o qual irá emitir luz (Weeks *et al.,* 1983; Yang *et al.,* 2002).

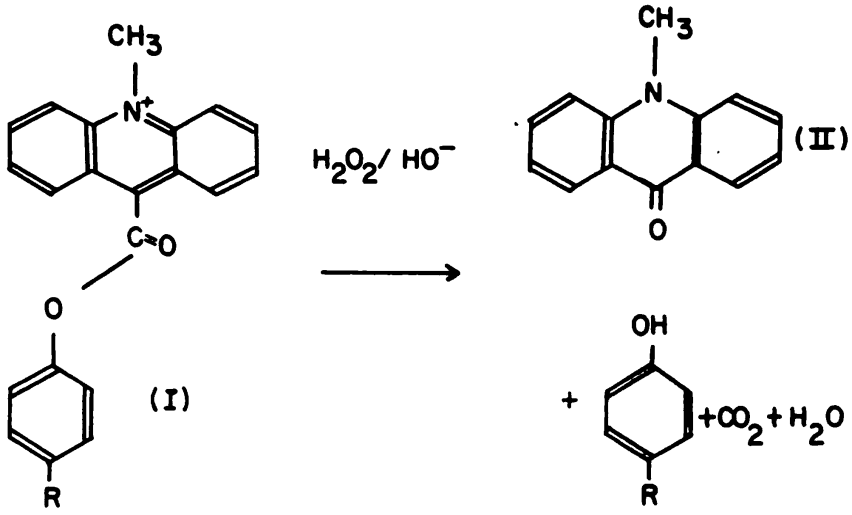

Figura 5. Reação Quimiluminescente do Ester de Acridina
(Fonte: Weeks *et al.,* 1983).

A utilização dos ésteres de acridina em imunoensaios quimiluminecentes foi promovida pela primeira vez por Weeks e colaboradores (1983) na preparação de anticorpos mono- ou policlonal marcado contra a alfa 1- fetoproteina, onde foi

demonstrado que este composto possuía uma alta sensibilidade de detecção, igual ou superior ao I^{125}.

Após conjugado a diversas moléculas, o éster de acridina tem sido empregado na preparação de sondas citoquímicas e histoquímicas. Assim como o éster de acridina, outros compostos, como o luminol e isoluminol, também são utilizados como marcadores em imunoensaios quimiluminescentes (Kricka, 2003).

Em nosso laboratório, foi testada a aplicação dos ensaios quimiluminescentes utilizando o éster de acridina conjugado à lectina concanavalina-A em lesões mamárias (Campos et al., 2006), e recentemente estão sendo testados ensaios imunoquimiluminescentes em lesões de próstata e tireóide com o anticorpo anti-galectina-3 conjugado ao ester de acridina, além de ensaios quimiluminescentes, com lectinas conjugadas ao ester de acridina, em amostras de pele que apresentam lesões tumorais (Araújo-Filho, 2011).

3. OBJETIVOS

3.1 Objetivo geral

Padronizar um protocolo para detecção de galectinas 3 a partir de anticorpos conjugados ao éster de acridina em tecidos neoplásicos da mama e comparar os resultados com a imunohistoquímica

3.2 Objetivos específicos

- Aprimorar um método de conjugação do éster de acridina aos anticorpos galectinas 3;

- Empregar quimiluminescência para quantificação de fótons (RLU) através da hidrólise do éster de acridina conjugado aos anticorpos;

- Comparar os resultados da expressão tecidual das galectina 3 com a imunohistoquímica através de reação enzimática (peroxidase) e reações de imunoquimiluminescência empregando conjugados de éster de acridina;

- Estabelecer o perfil de expressão imunohistoquímico das galectinas 3 entre os tumores de mama benignos, malignos e tecido normal;

4. RESULTADOS

CAPÍTULO I

CONJUGATION OF ANTI-GALECTIN-3 ANTIBODY TO ACRIDINIUM ESTER FOR CHEMILUMINESCENCE IMMUNOASSAY IN TISSUES

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CONJUGATION OF ANTI-GALECTIN-3 ANTIBODY TO ACRIDINIUM ESTER FOR CHEMILUMINESCENCE IMMUNOASSAY IN TISSUES

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ABSTRACT

The chemiluminescence, a process in which excited atoms or molecules through chemical reactions release their excess energy as light, have been the focus of research to broaden its application in various fields of biotechnology. This study aimed to conjugate the acridinium ester to antibody (anti-galectin3), to evaluate the shelf-life testing and promote chemiluminescent immunoassay in tumoral lesions of prostate and thyroid. Our results demonstrate the efficiency of conjugation of anti-galectin-3 and acridinium ester to a shelf-life with a recovery in 96.51% after 12 months. By applying this conjugate in chemiluminescent immunoassay on thyroid and prostate tissues, we found differential patterns between the studied lesions and their normal counterpart.

Keywords: acridinium ester, galectin-3, chemiluminescent immunoassay

1. Introduction

Chemiluminescence (CL) is defined as the emission of electromagnetic radiation, usually in the visible or near infrared, produced by a chemical reaction. This phenomenon has many applications in analytical chemistry, especially in the area of flow injection analysis, column chromatography and liquid separation systems by capillary electrophoresis as well as immunoassays [1].

The use of CL reactions has some advantages, among which have low detection limits and wide dynamic range [2]. The application of CL as an instrument of analysis depends on the coupling of the substance of interest to the participants of a chemiluminescent reaction or product in the excited state. The concentration of the unknown sample is proportional to the total production of light emitted or a physical parameter associated with the luminescence, such as color or polarization of light emitted [3].

Laboratory tests based on chemiluminescent principles have been developed for measurements requiring high sensitivity, for exemple, determination of cytokines, epidermal growth factor and vascular endothelial growth factor. Among the wide variety of tests are already commercially available to those used in the evaluation of thyroid

function, fertility, tumor markers, therapeutic drug monitoring, hepatitis, specific proteins and steroids [4].

Chemiluminescent substances can be detected in the range of fentomoles or atomoles (10^{-15} to 10^{-18} mol), with sensitivity superior to spectrophotometric assays (10^{-6} to 10^{-9} mol) and fluorometric (10^{-9} to 10^{-12} mol) [5]. Several organic compounds exhibit CL under appropriate conditions, among which stand out luminol, isoluminol, acridinium ester or its derivatives as some of the markers used in chemiluminescent immunoassays [2, 6].

The acridine is a fluorescent compound whose salts can be stimulated to produce light in the presence of hydrogen peroxide in dilute alkalis in the absence of a catalyst [3]. This property is due to the presence of a quaternary nitrogen center and derivatisation at carbon-9 which has a phenyl ester labeled a radical. Among the derivatives of acridine, acridinium ester received a special mention by the easy oxidation with hydrogen peroxide and the formation of an excited product (N-metilacridona), which will emit light [7, 8].

The acridinium ester (AE) was introduced in immunoassays from the need to replace the markers radiosotopic few that were becoming popular because of its short half life, danger to health and potential problems regarding the waste generated [7]. Since then the chemiluminescence of AE has been widely applied in analytical chemistry and biochemistry [9].

The low detection limits and background signal (without catalysts), the high quantum yield and easy conjugation to proteins, allowed the use of AE in many competitive immunoassays, non-competitive for the detection of several analytes at sub-picomolar: growth hormone, tumor markers (α-fetoprotein), interleukins, interferons, antibodies, proinsulin, and apolipoprotein B [10].

After several conjugated molecules, the acridinium ester has been used in the preparation of cytochemical and histochemical probes. Like some acridinium ester, other compounds such as luminol and isoluminol are also used as markers in chemiluminescent immunoassays [4].

This study proposes a combination between the acridinium ester and anti-galectin-3 to build a model of chemiluminescent immunoassay in tissues for differential diagnosis between glandular tumor lesions.

2. Materials and Methods

Characterization of acridinium Ester

Acridine derivatives emit light in the form of flash in a short period of time (3-5 seconds) after starting the chemical reaction. The chemical process involves the absorption of the chemiluminescence reagents, sufficient energy for the generation of an activated complex, which turns into an electronically excited product. The mechanism has been studied in detail by McCapra [11, 12, 13]. The most probable mechanism for the chemiluminescence reaction of AE is illustrated in Fig. 1. AE is stable in acid is rapidly hydrolyzed under alkaline conditions. In the presence of peroxide anion (HOO-) undergoes an AE intermolecular rearrangement forming the unstable intermediate dioxetanone. This compound decomposes rapidly producing N-metilacridona quimiluminesce at 470 nm. All intermediates, except the dioxetanone, have been isolated and characterized. McCapra has also proposed other routes that do not imply the dioxetanone [13].

Acridinium ester conjugation to anti-galectin3 (AE-AntiGal3)

The acridinium ester (Dimethyl Acridinium Ester (DMAE)-NHS/1966-1-53-2/Organic Lab kindly supplied by Dr. H. H. Weetall) was conjugated to the antibody according to the protocol developed by Weeks [7]. The antibody, anti-galectin-3 (Diagnostic Biosystems: clone 9C4, 500µl containing 2mg of protein) was incubated with 15µL of the AE solution (0.2 mg in 400µL of N,N-dimethylformamide) for 1 h at 25 °C under constant agitation. The conjugate (AE-AntiGal3) was applied to a column of Sephadex G-25 (10 x 1 cm) previously equilibrated with 10 mM phosphate buffer in 0.15 M NaCl (PBS), pH 7.2 (100ml). Aliquots (1 ml) were collected and the amount of protein determined spectrophotometrically at 280nm.

The chemiluminescence of the samples were measured by the luminometer Modulus Single Tube 9200-001 (Turner BioSystems) and the emission intensity was measured in relative light unit (RLU) using the following system: 50 µL of sample + 50µL H_2O_2 + 50µL NaOH. Protein concentration was determined according to Lowry [14].

3. Results and Discussion

Acridinium ester-Antibody conjugate

The conjugate was purified by molecular sieve chromatography (Sephadex G-25) where support resolved the sample into a single protein peak (ABS 280nm) eluted with PBS. We observed the same protein fractions of a peak luminescence (RLU), indicating the conjugation of the AE with antibody (Figure 1). The second peak represents the luminescence of free AE that did not bind to the protein. Ten repetitions were performed, obtaining always the same chromatographic pattern.

The easy coupling to protein using activated esters or imidates explain why the chemiluminogenic acridinium derivatives have been early proposed for ultrasensitive immunoassaysof thyroid stimulating hormone (TSH), immunoglobulins and related compounds [15]. Furthermore, conjugation of AE to antibodies and enzymes has been used in clinical and biomedical research [4, 16]. AE reacts with amino groups of proteins under mild conditions, forming a complex stable and reproducible. The marking procedure is simple for both proteins, as for peptides and low molecular weight molecules [1].

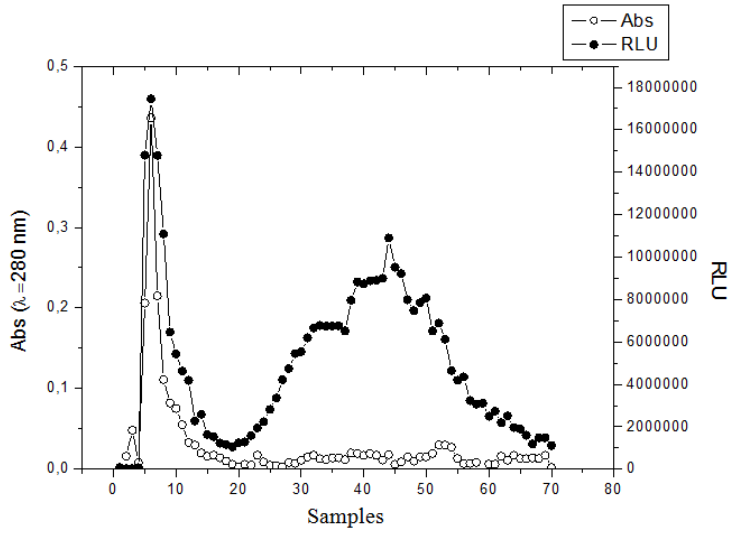

Figure 1: Chromatographic profile of the conjugate (AntiGal3-AE) on a column of Sephadex G-25 (10 x 1cm). Elution performed with 10 mM phosphate buffer in 0.15 M

NaCl (PBS), pH 7.2. Fractions (1ml samples) were collected and absorbance and chemiluminescence measured.

The conjugate (AE-AntiGal3) was placed in polypropylene test tubes and stored at -10°C. Its shelf life was investigated by running the emission of luminescence (RLU) and tested for stability according to different time intervals (1, 2, 3, 4, 5, 6 months and 12 months later).

The results demonstrated the stability of the conjugate compared to various time intervals. It was subjected to chemiluminescent assays where its percentage of recovery after a period of one year of storage remained in the range of 96.51% activity.

Application models

Despite all the interrelationships between performed immunohistochemistry with antibodies and tumor cells, there are few studies demonstrating the effectiveness of the use of chemiluminescent immunoassay in the marking of tissues with benign and malignant tumors.

Starting from the combination of acridinium ester with anti-galectin-3, chemiluminescents immunoassays were performed in tissues of prostate and thyroid carcinoma in order to test the immunogenicity of the antibody test to their respective tissue antigens.

Tissue samples of prostate and thyroid were selected, and after being subjected to routine histological complete, slices were obtained with 8um of thickness incubated with AE conjugated AntiGal3 and submitted to chemiluminescent immunoassay as described in Figure 2.

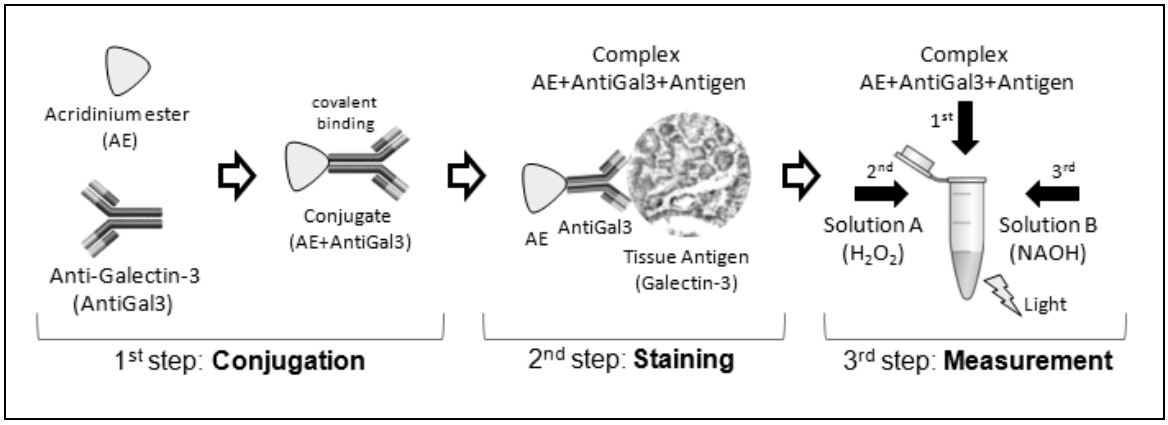

Figure 2. Representation of the steps used in the chemiluminescent immunoassay with AE-AntiGal3 in tissues.

Was standardized an average prostate tissue area (1.8 cm^2) and thyroid (2.5 cm^2), and the concentration of conjugate (100 µg / ml). After the chemiluminescent immunoassay, which occurred at approximately 135 min (incubation + measurement), it was possible to quantify the presence of galectin-3 in the lesions studied by photon emission, expressed as relative light units (RLU) during the breaking the link between the antibody and the ester (Table 1).

Table 1. Comparative profile of chemiluminescent immunoassay for anti-galectin-3 in tumor lesions of the thyroid and prostate.

Tissue	Lesion	Mean diameter (cm^2 tissue area)	Concentration of AE-AntiGal3	Reaction time (min)	Amplitude (RLU)
Prostate	Prostatic carcinoma	1,8	100 µg/ml	135	296.583
Thyroid	Carcinoma Papillary	2,5	100 µg/ml	135	942.190

Inhibition 6.869 RLU (prostate carcinoma), 15.381 RLU (carcinoma papillary thyroid)

These results corroborate those reported in the literature with respect to conventional immunohistochemistry, where is observed an increaded presence of galectin in papillary thyroid carcinoma [17, 18] and a decrease of this expression in prostatic carcinoma [19, 20].

This evidence find that the chemiluminescent immunoassay technique has a high sensitivity and specificity stimulating investigations on the use of antibodies conjugated to chemiluminescent markers for the diagnosis of neoplasms.

4. Conclusions

In conclusion, the combination of acridinium ester to antibody (AE-AntiGal3) proved to be a simple procedure and can be easily repeated.

From these data could be observed that the combination of AE-AntiGal3 can be stored for an average period of 12 months, maintaining good capacity to generate chemiluminescent reactions.

Our tests in lesions of the prostate and thyroid tumors showed that the tissue analysis using chemiluminescent immunoassay with specific markers, may be a valuable tool, used in association with others procediments, in the differential diagnosis of tumors.

These results demonstrate the stability of the conjugate studied, and adds to it, prospects as regards its use in the manufacture of diagnostic kits, for use in the diagnosis of tumor lesions.

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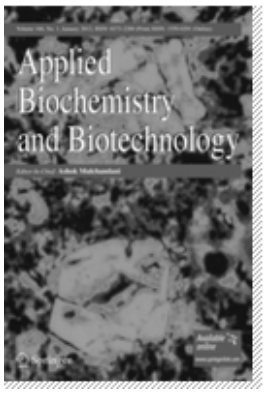
Conflict of interest

We declare that none of the authors have a financial interest related to this work.

CAPÍTULO II

CHEMILUMINESCENT DETECTION OF GALECTIN-3 IN BREAST TUMORAL TISSUES.



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CHEMILUMINESCENT DETECTION OF GALECTIN-3 IN BREAST TUMORAL TISSUES.

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Abstract Galectin-3 (Gal3) is involved in immune system and cancer. Immunohistochemical methods to detect it and based on qualitative analysis present disparity and variability depending on the observers. This contribution proposes a chemiluminescent quantitative method. Monoclonal antibody anti-Gal3 was conjugated to acridinium ester (AE) and the complex formed with Gal3 in the breast tissue was chemiluminescently detected. The light emission (expressed in Relative Light Unit – RLU) showed mean values higher in fibroadenoma and normal tissues than in IDC). These values showed to be statistically significant ($p<0.001$). There was a linear relationship between RLU and tissue area. Furthermore, these values were dramatically reduced when the tissue samples were previously incubated with non labeled anti-Gal3. Finally, the anti-Gal3-AE solution in buffer stored at 4oC and the treated samples showed to be stable during a year and at least 72 h, respectively. Gal3 content in breast tissue followed the pattern: Fibroadenoma>normal>IDC. This quantitative, specific and sensitive method based on labeling antibody to acridinium ester can be applied to detect antigen in tissue. Breast tissues were used as a model.

Keywords: Chemiluminescence, galectin 3, Human mammary tissues, breast cancer.

Introduction

Breast cancer is the second most frequent neoplasia in women as well as the main cause of death. Advances in investigations of the molecular changes happening in cells under malignant transformation can help us to understand the mechanisms of the occurrence and progression of tumors [1].

The search for specific molecules associated with carcinogenic process has led to a growing number of possible biomarkers. Associated with clinical and traditional histological parameters they have been used to identify high risk patient groups [2, 3].

According to the American Society of Clinical Oncology (ASCO) thirteen categories of breast tumor markers were considered such as carcinoembryonic antigen, estrogen and progesterone receptors, human epidermal growth factor receptor 2, urokinase plasminogen activator, plasminogen activator inhibitor 1 and certain multiparameter gene expression assays; but among them some are not clinically applied [4].

The expression of galectin-3 (Gal-3) has been described to decrease in situ breast carcinoma and this down-regulation was more pronounced in invasive ductal carcinoma (IDC), particularly when associated with infiltration of axillary lymph nodes [5]. Galectins are a family of β-galactoside binding lectins that are multifunctional proteins involved in a variety of biological processes Results such as growth development, immune functions, apoptosis, and cancer metastasis [6]. They have been widely used as

tools to describe immunohistochemical changes in the tumor cell surface, probably, by its influence on apoptosis or metastasis [7]. Gal3 is one of the best studied galectins with many proposed functions in the immune system and cancer [8]. It has a small molecular weight (about 30 kDa) protein that is expressed in many types of human cells, in particular epithelial and immune cells [9]. Gal3 is highly expressed in normal but not in malignant cells [10].

There are currently several diagnostic techniques that assist in treatment of cancers, including the immunohistochemical tissue biopsies. They are of great help in discriminating similar tumors that are often difficult to interpret by the routine histological methods [11, 12, 13].

The chemiluminescence method, the generation of light via a chemical reaction, has gained widespread use as a detection mode over the last several years [14]. The method offers a significant combination of advantages in terms of sensitivity and reagent stability; a wide range of detection methods (from photographic film to sophisticated instrumentation) and low hazard compared to other isotopic and non-isotopic detection methods employed in experimental and traditional assays [15]. Consequently, chemiluminescent methods are now established in clinical analysis routine and as a tool in clinical and biomedical research [14].

The aim of this study was to identify the Gal3 in benign and malignant breast tissues by using a specific antibody labeled to acridinium ester (AE) .

Methods

Human mammary specimens.

Samples of breast tissues were obtained from women (mean age of 62.5 years old) and submitted to formalin-fixed and paraffin-embedded. The tumors were diagnosed as fibroadenoma (n=35) and IDC (n=35) were obtained from the tissue bank of the Clinic Hospital of the Federal University of Pernambuco – UFPE, Northeast Brazil. Clinical staging of 3 for all IDC samples was defined according to the TNM Classification of malignant tumors [16]. Fifteen samples of normal breast tissues were collected from a plastic surgery clinic as control. All tissue samples were histologically processed, embedded in paraffin and hematoxylin and eosin stained.

Anti-Gal3 antibody conjugation with acridinium ester.

AE was conjugated to anti-Gal3 antibody (Diagnostic Biosystems: clone 9C4) according to Campbell et al. [17]. Briefly, anti-Gal3 (500 µl containing 2 mg of protein) was incubated with 15 µl of AE solution (0.2 mg diluted in 400 µl of N,N-dimethylformamide) for 1 h at 25^{o}C. The conjugate (anti-Gal3-AE complex) was applied to a column of Sephadex G-25 (10 x 1 cm), previously equilibrated with 10 mM phosphate buffer, pH 7.2 (100 ml) and eluted with this buffer. Aliquots (1 ml) were collected and their protein content was spectrophotometrically determined at 280 nm and chemiluminescence assayed. The aliquots containing protein and chemiluminescence were pooled, dialyzed overnight against 10 mM phosphate buffer, pH 7.2, and kept at -10^{o}C until use.

Chemiluminescent immunoassay.

Paraffin sections (8 µm) of mammary specimens were cut, transferred to glass slides, deparaffinized in xylene (1st for 5 min and 10 dips in 4 successive containers with xylene) and hydrated in graded alcohols (3 x 100% and 1 x 70% - 10 dips each). Afterwards

tissue slices were incubated with anti-gal3-AE (100 µL - 100 mg mL^{-1}) for 2 h at 4 °C, followed by washings (2 x 5 min) with 5 mL of 10 mM phosphate buffer, containing 0.15 M NaCl (PBS), pH 7.2. Then the tissues were transferred to polypropylene test tubes with a volume of 50 µL of PBS. Finally, solutions of 0.5% H_2O_2 in 0.1 N HNO_3 (50 µl) and 0.25 M NaOH (50 µl) were added for chemiluminescent measurement using a luminometer Modulus Single Tube 9200-001 (Turner BioSystems, USA).
The emission intensity was determined as relative light units (RLU) with a counting time of 5 seconds per sample. Anti-Gal3-AE binding inhibition assays were accomplished by incubating the tissues with non labeled Anti-Gal3 (100 µL - 100 mg mL^{-1}) for 2 h at 4 °C prior to its incubation with anti-Gal3-AE.

Statistical analysis.
The software GraphPad Prism 5 was used for the chemiluminescence (number of RLU per sample) data processing (expressed as mean ± standard deviation) and statistical analysis (Tukey-test and Student t-test; $p<0.05$).

Results and Discussion

In the present study the AE was conjugated to anti-galectin-3 and was collected by Sephadex G-25 column chromatography. Elution was carried out with 10 mM phosphate buffer, pH 7.2. Fractions (aliquots of 1 ml) were collected and subject to measures in the luminometer. The conjugate was found around the 6th fraction and showed to be very stable yielding RLU values of 96.51 ± 3 % during a year.
The anti-Gal3-AE conjugate used to identify the Gal3 in breast tissues. Figure 1 shows the RLU mean values obtained for normal (58.87 x 104 ± 17880), IDC (39.8 x 104 ± 29344) and fibroadenoma (88.88 x 104 ± 17880). The difference among these mean values showed to be statistically significant ($p<0.001$). Furthermore, these values were dramatically reduced when the tissue samples were previously incubated with non labeled anti-Gal3 antibody yielding values of 2307.25 ± 496.00 RLU (normal); 2185.66 ± 637 RLU (IDC) and 2507.66 ± 969 RLU (Fibroadenoma).

The reduced expression of Gal-3 has been described in breast cancer and was associated with increasing histological grade. This report suggests that this reduction can be related with the acquisition of invasive and metastatic potential which possibly resulted from reduced extracellular matrix binding and increased cell motility [18]. Similarly, expression of Gal-3 is down-regulated in prostate [19] and ovarian cancers [20].
Historically, luminol and isoluminol were the chemiluminescent labels of choice but they became obsolete by the advantages of AE such as high inherent sensitivity, low quenching, simplicity and easy of handling and disposal and a long shelf-life [21]. The AE molecule rapidly reacts (typically 1 to 5 sec) with hydrogen peroxide under alkaline conditions to produce light at 430 nm. These rapid reaction kinetics permit detection over a very short time frame, thereby minimizing background noise and improving overall sensitivity. Detection in a standard luminometer exhibits a linear response over an AE concentration range of more than 4 orders of magnitude, with a detection limit of approximately 5 x 10 -19 mole [22].
In our lab, chemiluminescence has been used to reveal the glycocode of breast tissues . Concanavalin A was labeled with AE and used to evaluate biopsies of normal and IDC. RLU values were much higher for the IDC than the normal tissue (about 500 times

higher). The work also demonstrated that RLU presented a linear relationship with the labeled tissue area [23].
Breast Fibroadenoma is a common benign tumor composed of epithelial and stromal components that usually occurs in young women and has rarely been associated with the development of malignancy. On the other hand, the histological diagnosis of carcinoma sometimes could be similar to fibroadenoma. This observation has been based on the criterion that the carcinoma cells were limited to a well-defined fibroadenoma or only focally extended into adjacent stroma or ducts [24].
Tumor progression is related to molecular changes in cells under malignant transformation [25]. The search for new neoplasic biomarkers in association with clinical and traditional histological parameters has been used to identify patients with high risk and aggressive clinical course of disease [3,25].

Conclusions

From the results above one can conclude that there was a statistically significant reduction in tissue expression of Gal3 in breast IDC cells compared to normal tissue. Fibroadenoma showed a significant increase in cellular expression of Gal3 when compared with the malignant and normal tissue. The anti-Gal3-AE conjugate and the treated samples showed to be stables for one year and 72 h, respectively. This study also demonstrated that imunochemiluminescence can be valuable in tissue analysis, showing high sensitivity and specificity. Thus the antibodies conjugated to AE can be used as quantitative tool, minimizing the subjectivity of the analysis.

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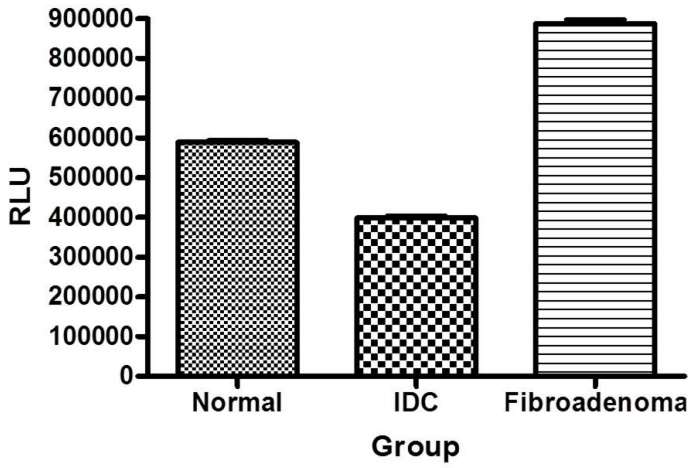

Fig. 1 Chemiluminescent immunoassay. The light emission (RLU) from normal, Fibroadenoma and infiltrative ductal carcinoma (IDC) . The statistical analyses showed significance between the mean values ($p<0.001$).

CAPÍTULO III

COMPARISON BETWEEN IMMUNOCHEMILUMINESCENCE AND IMMUNOHISTOCHEMISTRY OF GALECTIN-3 IN BREAST TUMORS

AIMM Applied Immunohistochemistry & Molecular Morphology
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Impact factor: 1.709

Comparison between immunochemiluminescence and immunohistochemistry of Galectin-3 in breast tumors

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Abstract

Galectin-3 (Gal3) is involved in immune system and cancer. Immunohistochemical methods to detect it and based on qualitative analysis present disparity and variability depending on the observers. This contribution proposes a chemiluminescent quantitative method. Monoclonal antibody anti-Gal3 was conjugated to acridinium ester (AE) and the complex formed with Gal3 in the breast tissue was chemiluminescently detected**.** The Immunohistochemistry was developed using monoclonal anti-galectin-3 antibody. Slices of the same samples from different patients with invasive ductal carcinoma (IDC) (n = 35), FA (n = 35) and N (n = 15) were fixed in formalin, processed and embedded in paraffin. Tissue slices (5µm) were incubated with anti-galectin-3 antibody solution for one hour at room temperature and then incubated with a secondary anti-body conjugated to peroxidase. The stain pattern was visualized with diaminobenzidine (DAB) and hydrogen peroxide. Image analysis was carried out using a workstation consisting of a standard light microscope equipped with a digitalizing camera connected to a desktop personal computer. The light emission and immunohistochemestry (expressed in Relative Light Unit – RLU and cells marked for field) showed mean values higher in fibroadenoma and normal tissues than in IDC. These values showed to be statistically significant ($p<0.001$). This quantitative, specific and sensitive method based on labeling antibody to acridinium ester can be applied to detect antigen in tissue. Breast tissues were used as a model.

Key words: Immunochemiluminescence**,** Immunohistochemistry, galectin 3, breast tumors, image analysis

Introduction

There are currently several diagnostic techniques that assist in treatment of cancers, including the immunohistochemical tissue biopsies. They are of great help in discriminating similar tumors that are often difficult to interpret by the routine histological methods [1, 2, 3].

The chemiluminescence method, the generation of light via a chemical reaction, has gained widespread use as a detection mode over the last several years[4]. The method offers a significant combination of advantages in terms of sensitivity and reagent stability; a wide range of detection methods (from photographic film to sophisticated instrumentation) and low hazard compared to other isotopic and non-isotopic detection methods employed in experimental and traditional assays[5]. Consequently, chemiluminescent methods are now established in clinical analysis routine and as a tool in clinical and biomedical research[4].

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The aim of this study was to identify the Gal3 in benign and malignant breast tissues by using a specific antibody labeled to acridinium ester (AE) and compare with the immunohistochemistry for the same antigen.

Materials and methods

Human mammary specimens.

Samples of breast tissues were obtained from women (mean age of 62.5 years old) and submitted to formalin-fixed and paraffin-embedded. The tumors were diagnosed as fibroadenoma (n=35) and IDC (n=35) were obtained from the tissue bank of the Clinic Hospital of the Federal University of Pernambuco – UFPE, Northeast Brazil. Clinical staging of 3 for all IDC samples was defined according to the TNM Classification of malignant tumors[16]. Fifteen samples of normal breast tissues were collected from a plastic surgery clinic as control. All tissue samples were histologically processed, embedded in paraffin and hematoxylin and eosin stained.

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Chemiluminescent immunoassay.

Paraffin sections (8 μm) of mammary specimens were cut, transferred to glass slides, deparaffinized in xylene (1st for 5 min and 10 dips in 4 successive containers with xylene) and hydrated in graded alcohols (3 x 100% and 1 x 70% - 10 dips each). Afterwards tissue slices were incubated with anti-gal3-AE (100 μL - 100 mg mL-1) for 2 h at 4 °C, followed by washings (2 x 5 min) with 5 mL of 10 mM phosphate buffer, containing 0.15 M NaCl (PBS), pH 7.2. Then the tissues were transferred to polypropylene test tubes with a volume of 50 μL of PBS. Finally, solutions of 0.5% H2O2 in 0.1 N HNO3 (50 μl) and 0.25 M NaOH (50 μl) were added for chemiluminescent measurement using a luminometer Modulus Single Tube 9200-001 (Turner BioSystems, USA).

The emission intensity was determined as relative light units (RLU) with a counting time of 5 seconds per sample. Anti-Gal3-AE binding inhibition assays were accomplished by incubating the tissues with non labeled Anti-Gal3 (100 μL - 100 mg mL-1) for 2 h at 4 °C prior to its incubation with anti-Gal3-AE.

Immunohistochemical assay

The immunohistochemical demonstration of Gal-3 was carried out by using an antigen retrieval method as we have described earlier with the mouse monoclonal antibody NCL-Gal-3 (clone 9C4; Leica Biosystems, Newcastle, UK). In brief, 5-μm sections were cut sequentially and mounted onto superfrost-treated slides (Menzel-Glasse, Braunschweig, Germany). The slides were then dried overnight at 37ºC before deparaffinization in

xylene and rehydratation through graded ethanols. For epitope retrieval, the slides were immersed in a waterbath at 95ºC for 44 minutes with a EDTA buffer, pH 9.0. Then, the slides were cooled in the buffer for 20 minutes at room temperature. Hydrogen peroxide (0, 3% was added to the slides and incubated at room temperature for 30 minutes. The tissues were then incubated for 1 hour with the primary antibody (with dilution of 1:50) always at room temperature. The Novolink Polymer Detection System (Leica Biosystems) was used for the subsequent steps according to the manufacturer's instructions. Chromogenic development was accomplished using diaminobenzidine hydrogen peroxide. The slides were then slightly counterstained with hematoxylin and dehydrated, and a cover slip was applied. Controls for nonspecific binding of the primary antibody preparations were substituted as the first layer of serial sections. Gal-3 immunoreactivity was scored for cytoplasmic/membranous and nuclear staining (figure 1). Cells marked were selected according to the method developed by Dowsett et. al, 2003[18].

Digital image analysis

Images of slices of the stained cells (magnification 40x) were obtained using a digital video camera (Sony, Japan) connected to a microscope and processed using the free software Image j, version 1.46. Five fields were chosen randomly from various section levels to ensure objectivity of sampling. The files were then opened in image j and further processed to assess stained cells. Cells marked were counted in each field irrespective of marking be nuclear, cytoplasmic or in membrane.

Statistical analysis.

The software GraphPad Prism 5 was used for the chemiluminescence (number of RLU per sample) data processing (expressed as mean ± standard deviation) and for the immunohistochemistry (number of cells for field) data processing (expressed as mean ± standard deviation) and statistical analysis (Tukey-test and Student t-test; $p<0.05$).

RESULTS

The immunohistochemical staining studies demonstrated significant differences in the patterns of Gal-3 expression in the tissues (Figure 2). The fibroadenoma (FA) showed a higher number of cells marked by area than the IDC cells and normal breast tissue. however fibroadenoma and normal tissue showed no significant differences between them.

The anti-Gal3-AE conjugate used to identify the Gal3 in breast tissues shows the RLU mean values obtained for normal (58.87 x 104 ± 17880), IDC (39.8 x 104 ± 29344) and fibroadenoma (88.88 x 104 ± 17880). The difference among these mean values showed to be statistically significant ($p<0.001$). Furthermore, these values were dramatically reduced when the tissue samples were previously incubated with non labeled anti-Gal3 antibody yielding values of 2307.25 ± 496.00 RLU (normal); 2185.66 ± 637 RLU (IDC) and 2507.66 ± 969 RLU (Fibroadenoma).

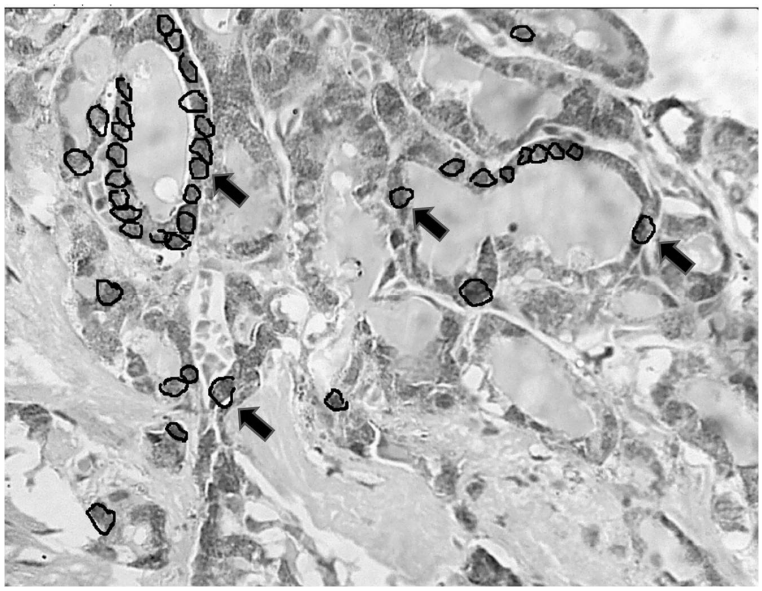

Fig. 1 Digital image analysis. Arrows show cells marked with Gal-3 circulated by Image j tool in IDC. that were later counted 4 fields per sample (magnification 400X).

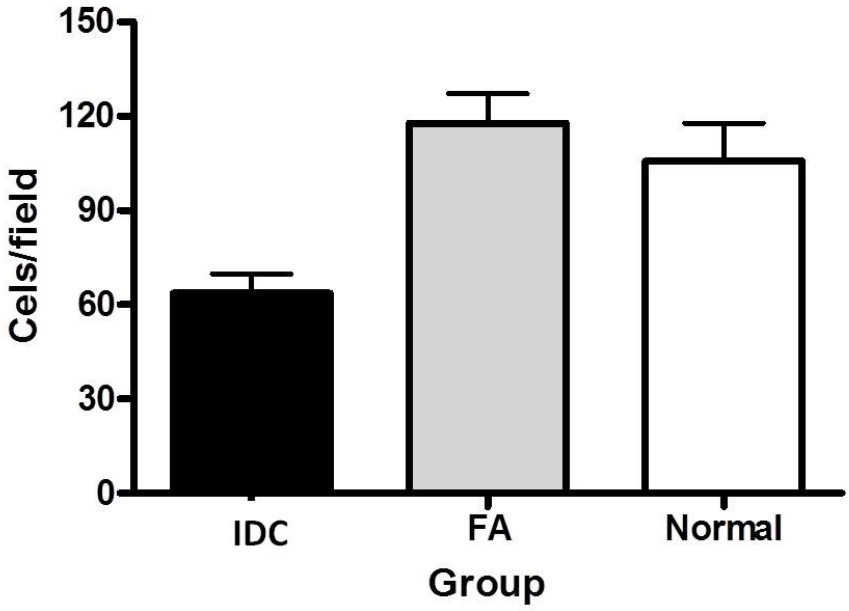

Fig. 2 Immunohistochemical staining. The fibroadenoma (FA) showed a higher number of cells marked by area than the IDC cells and normal breast tissue. However fibroadenoma and normal tissue showed no significant differences between them.

DISCUSSION

The reduced expression of Gal-3 has been described in breast cancer and was associated with increasing histological grade. This report suggests that this reduction can be related with the acquisition of invasive and metastatic potential which possibly resulted from reduced extracellular matrix binding and increased cell motility[19]. Similarly, expression of Gal-3 is down-regulated in prostate [20] and ovarian cancers [21].

Historically, luminol and isoluminol were the chemiluminescent labels of choice but they became obsolete by the advantages of AE such as high inherent sensitivity, low quenching, simplicity and easy of handling and disposal and a long shelf-life [22]. The AE molecule rapidly reacts (typically 1 to 5 sec) with hydrogen peroxide under alkaline conditions to produce light at 430 nm. These rapid reaction kinetics permit detection over a very short time frame, thereby minimizing background noise and improving overall sensitivity. Detection in a standard luminometer exhibits a linear response over an AE concentration range of more than 4 orders of magnitude, with a detection limit of approximately 5 x 10 -19 mole [23].

In our lab, chemiluminescence has been used to reveal the glycocode of breast tissues . Concanavalin A was labeled with AE and used to evaluate biopsies of normal and IDC. RLU values were much higher for the IDC than the normal tissue (about 500 times higher). The work also demonstrated that RLU presented a linear relationship with the labeled tissue area [24].

Breast Fibroadenoma is a common benign tumor composed of epithelial and stromal components that usually occurs in young women and has rarely been associated with the development of malignancy. On the other hand, the histological diagnosis of carcinoma sometimes could be similar to fibroadenoma. This observation has been based on the

criterion that the carcinoma cells were limited to a well-defined fibroadenoma or only focally extended into adjacent stroma or ducts [25].

Tumor progression is related to molecular changes in cells under malignant transformation[26]. The search for new neoplasic biomarkers in association with clinical and traditional histological parameters has been used to identify patients with high risk and aggressive clinical course of disease [8,27].

The IHC is usually the first technique performed to confirm diagnosis of cancer, with the following possible outcomes: score 0 (no marking or labeling of the cytoplasmic membrane <10% neoplastic cells), score 1 (incomplete marking and Mild em> 10% of neoplastic cells, score 2 + (markup mild to moderate, the entire membrane in> 10% of neoplastic cells), score 3 + (strong labeling of the entire membrane in> 10% of neoplastic cells. The cases with score 0 and 1 + are considered negative. [26,27]

With consequent difference between sensitivity and specificity rivers of the antibodies used go-between different antigen retrieval procedures and between dilutions of the antibody, associated with some subjective reading of results, lead to this method is great variability, responsible for some amplitude at percentage of positive invasive carcinomas, as reported in the literature ranging from 15 to 30% [28].

CONCLUSIONS

This method demonstrated that imunochemiluminescence can be value in tissue analysis, showing high sensitivity and perspective of application in early diagnosis due to its detection at the molecular level. Thus the antibodies conjugated to acridinium ester can be used as tool to quantify the changes, minimizing the subjectivity of the analysis. From the results above one can conclude that there was a statistically significant reduction in tissue expression of Gal3 in brest IDC cells compared to normal tissue. Fibroadenoma showed a

significant increase in cellular expression of Gal3 when compared with the counterpart malignant and normal tissue. The anti-Gal3-AE conjugate and the treated samples showed to be stables for one year and 72 h, respectively.

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5. CONCLUSÕES

A partir dos resultados obtidos, podemos concluir que:

- A conjugação do éster de acridina ao anticorpo (EA-AntiGal3) demonstrou ser um procedimento que pode ser repetido, o conjugado pode ser armazenado por um período médio de 12 meses, mantendo boa capacidade para gerar reações quimiluminescentes;
- No tecido mamário os resultados da imunoquimiluminescência foram semelhantes aos da imunoistoquímica, que demonstraram uma redução estatisticamente significante na expressão tecidual da galectina-3 em células de carcinoma ductal invasivo quando comparado com tecido normal, e um aumento dessa expressão no fibroadenoma quando comparado com os tecidos malignos e normais;
- O conjugado EA-AntiGal3 previamente incubados nos tecidos apresentou estabilidade quando submetido a imunoquimiluminescência mesmo após um período de 72 h;
- A imunoquimiluminescência apresentou resultados reprodutíveis na análise de tecidos, mostrando alta sensibilidade e a perspectiva de aplicação no diagnóstico precoce de lesões tumorais de mama devido à sua detecção em nível molecular.

6. PERSPECTIVAS

- Imobilizar outros marcadores tumorais ao éster de acridina para a realização de ensaios imunoquimiluminescentes em lesões tumorais da mama e compará-las com os métodos imunohistoquímicos a fim de validar a eficácia da metodologia desenvolvida quando comparada às técnicas utilizadas na rotina;

- Desenvolver estratégias metodológicas para a marcação de células tumorais de tecidos que não tenham passado pela rotina histológica (frescos) para possíveis testes de diagnóstico diferencial das lesões tumorais com uma maior rapidez;

- Promover a interface entre a imunoquimiluminescência em tecidos com patologistas, a fim de inserir a técnica na rotina do diagnóstico.

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[3] U. Neugebauer, P. Rösch, M. Schmitt, J. Popp, C. Julien, A. Rasmussen, C. Budich, and V. Deckert, ChemPhysChem **7**, 1428-1430 (2006).
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[4] B. J. Ansell, in: Proceedings of the 4th International Conference on Nitride Semiconductors, Denver, Colo-rado, USA, 16-20 July 2001, Part B.1.
[5] V. Piatnitsa, D. Kholodnyak, P. Kapitanova, I. Fischuk, T. Tick, J. Jantti, H. Jantunen, and I. Vendik, in: Proceed-ings of the 37th European Microwave Conference, Munich, Germany, October 2007, pp. 636-639.

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[8] A. Studentname, PhD thesis, University of Newcastle (UK), 1991.

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5. Friedman SA. Preeclampsia: a review of the role of prostaglandins. Obstet Gynecol [serial online]. January 1988;71:22 37. Available from: BRS Information Technologies, McLean, VA. Accessed December 15, 1990.

Database
6. CANCERNET-PDQ [database online]. Bethesda, MD: National Cancer Institute; 1996. Updated March 29, 1996.

World Wide Web
7. Gostin LO. Drug use and HIV/AIDS [*JAMA* HIV/AIDS web site]. June 1, 1996. Available at: http://www.ama-assn.org/special/hiv/ethics. Accessed June 26, 1997.

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