UNIVERSIDADE FEDERAL DE UBERLÂNDIA
INSTITUTO DE GENÉTICA E BIOQUÍMICA
PÓS-GRADUAÇÃO EM GENÉTICA E BIOQUÍMICA

IMPLICAÇÕES CLÍNICAS DE UM ANTICORPO RECOMBINANTE (FAB) CONSTRUÍDO E SELECIONADO POR PHAGE DISPLAY E AVALIAÇÃO DO PAPEL DAS CITOQUERATINAS NO CÂNCER DE MAMA

ALUNA: THAISE GONÇALVES DE ARAÚJO

ORIENTADOR: PROF. DR. LUIZ RICARDO GOULART FILHO

UBERLÂNDIA – MG
2012

UNIVERSIDADE FEDERAL DE UBERLÂNDIA
INSTITUTO DE GENÉTICA E BIOQUÍMICA
PÓS-GRADUAÇÃO EM GENÉTICA E BIOQUÍMICA

IMPLICAÇÕES CLÍNICAS DE UM ANTICORPO RECOMBINANTE (FAB) CONSTRUÍDO E SELECIONADO POR PHAGE DISPLAY E AVALIAÇÃO DO PAPEL DAS CITOQUERATINAS NO CÂNCER DE MAMA

ALUNA: THAISE GONÇALVES DE ARAÚJO

ORIENTADOR: PROF. DR. LUIZ RICARDO GOULART FILHO

Tese apresentada à Universidade Federal de Uberlândia como parte dos requisitos para obtenção do Título de Doutor em Genética e Bioquímica (Área Genética)

UBERLÂNDIA – MG

2012

Dados Internacionais de Catalogação na Publicação (CIP)

Sistema de Bibliotecas da UFU, MG, Brasil.

A659i
2012

Araújo, Thaise Gonçalves de, 1984-

Implicações clínicas de um anticorpo recombinante (Fab) construído e selecionado por *Phage display* e avaliação do papel das citoqueratinas no câncer de mama / Thaise Gonçalves de Araújo. -- 2012.

114 f.

Orientador: Luiz Ricardo Goulart Filho.

Tese (doutorado) - Universidade Federal de Uberlândia, Programa de Pós-Graduação em Genética e Bioquímica

Inclui bibliografia.

1. Genética - Teses. 2. Genética molecular - Teses. 3. Mamas Câncer - Teses. I. Goulart Filho, Luiz Ricardo, 1962- . II. Universidade Federal de Uberlândia. Programa de Pós-Graduação em Genética e Bioquímica. III. Título.

CDU: 575

UNIVERSIDADE FEDERAL DE UBERLÂNDIA
INSTITUTO DE GENÉTICA E BIOQUÍMICA
PÓS-GRADUAÇÃO EM GENÉTICA E BIOQUÍMICA

IMPLICAÇÕES CLÍNICAS DE UM ANTICORPO RECOMBINANTE (FAB) CONSTRUÍDO E SELECIONADO POR PHAGE DISPLAY E AVALIAÇÃO DO PAPEL DAS CITOQUERATINAS NO CÂNCER DE MAMA

ALUNA: THAISE GONÇALVES DE ARAÚJO

COMISSÃO EXAMINADORA

PRESIDENTE: LUIZ RICARDO GOULART FILHO (ORIENTADOR)

Examinadores:

Dr. Carlos Eduardo Paiva (Hospital de Câncer de Barretos)
Dra. Adriana Freitas Neves (Universidade Federal de Goiás)
Dr. Jair Pereira da Cunha Júnior (Universidade Federal de Uberlândia)
Dr. Marcelo José Barbosa Silva (Universidade Federal de Uberlândia)

Data de Defesa: 19/12/2012

As sugestões da Comissão Examinadora e as Normas PGGB para o formato da Tese foram contempladas

_____

Prof. Dr. Luiz Ricardo Goulart

"A vida é um acontecimento que merece ser comemorado. A cada dia, a cada instante, ela se renova generosa nos pequenos espaços. A vida é miúda, feita de pequenas partes. Viver é construir um mosaico, parte por parte, dia após dia. A beleza de um momento unida à tristeza de outras horas passa a ocupar o mesmo espaço no quadro. As cores se misturam e se arquitetam em busca da harmonia tão desejada. Há dias em que as cores são frias [...]

A vida pede calma, silêncio, pausas [...]

Há dias em que as cores são quentes [...]

A vida rompe com toda forma de calma [...]

Não suportaríamos permanecer em um só lado dessas possibilidades!"

Pe. Fábio de Melo

DEDICATÓRIA

Dedico essa conquista, primeiramente a Deus, meu Pai protetor.

Aos meus pais, Itamar e Márcia: Amo muito vocês!!

À minha Tia Irami, que me ensinou que, para aqueles que têm fé, nada, nada é impossível!!!

AGRADECIMENTOS

Primeiramente, gostaria de agradecer ao Prof. Dr. Luiz Ricardo, meu grande mentor e mestre. Muito obrigada pelas oportunidades, pela confiança, pela amizade. Serei eternamente grata!!!

À Dra. Adriana Freitas Neves, que, definitivamente, palavras não existem para agradecer tudo o que tem feito por mim. É uma grande amizade que levarei para toda a vida!

À Yarinha, amiga e colega guerreira. Só nós duas sabemos o que já passamos com viagens, experimentos e teses. Grandes amizades são construídas assim, quando dividimos nosso cotidiano e nos identificamos nos problemas.

Ao André, que me ensinou que jamais devemos desistir de nossos sonhos e que nada é impossível quando lutamos e desejamos muito que tudo se realize... Adoro demais!! (No matter what!!)

À minha amiga Ju!! Pelos momentos divididos, pelos conselhos, pelas conversas, por tudo! Adoro muito!!!!

À Karina, que me ensinou que grandes amizades resistem a grandes turbilhões e se fortalecem com eles. Saiba que sempre estarei aqui quando precisar!

À Claudinha, com quem eu pude contar nos momentos e experimentos finais. Com certeza colheremos muitos frutos dessa parceria que, claro, virou uma grande amizade.

Ao meu amigo Galbitcho! Te adoro muito!

À Patrícia Terra, que além de aluna é amiga, e que sei que poderei sempre contar! Torço muito por você!

À Paula Souza, uma amizade que foi aparecendo de mansinho... e que hoje vale muito a pena cultivar!

Aos grandes amigos da família Nanos (não vou descrever cada um para não correr o risco de esquecer alguém). Saibam que crescemos juntos e que criamos laços para toda uma vida!! Amo todos vocês!!!

Ao Prof. Dr. Carlos Ueira Vieira pelos conselhos, pelos ensinamentos e pelos momentos compartilhados.

Às minhas eternas amigas Danielle, Bel e Patrícia. Confidentes e que, apesar da distância sei que posso sempre contar com o carinho e ajuda! Saudades imensas!

À minha família, primos e tios por toda a torcida e carinho e aos meus primos-irmãos Juninho, Cy e Beta (Lorenzo também). Certeza de carinho e apoio. Amo vocês!!!

À Cici, minha segunda mãe e à Dona Alice, pela certeza do apoio e orações.

Aos docentes do Instituto de Genética e Bioquímica pelos ensinamentos ministrados e pela cooperação.

Aos secretários do INGEB Gerson e Madison, pela paciência e pelos serviços prestados.

À equipe médica do Hospital de Clínicas da Universidade Federal de Uberlândia: Dr. Donizete, Dra. Luanda, Dra. Priscila, Dr. Thales, Dr. Alair, Dr. Sinval e Dr. Eduardo, Emília, Noêmia, Osmar, Vanessa, Gina, Cleonice, Aparecida, Maria de Fátima, Ana Aloisa, Divina. Sem o trabalho e compreensão de vocês nenhum resultado seria alcançado. Muito obrigada!

Às pacientes, que apesar de toda a dor e sofrimento contribuíram diretamente para a realização desse estudo.

A Capes, financiadora do meu trabalho.

Ao Laboratório de Nanobiotecnologia, meu trabalho e grande responsável pela minha formação profissional.

SUMÁRIO

LISTA DE FIGURAS

Capítulo IV: Cytokeratins' transcripts as predictors of breast cancer molecular subtypes

LISTA DE TABELAS

LISTA DE ABREVIATURAS E SÍMBOLOS

A	Adenina
ADCC	Citotoxicidade celular dependente de anticorpo
BC	*Breast Cancer*
BSA	Soroalbumina bovina
C	Citosina
ºC	Graus Celsius
CDC	Citotoxicidade dependente do complemento
cDNA	Ácido Desoxirribonucléico complementar
CDR	Região determinante de complementariedade
CH	Domínio Constante de cadeia pesada
CID-O	Classificação Internacional de Doenças para Oncologia
CK	Citoqueratina
CL	Domínio Constante de cadeia leve
CM	Câncer de Mama
cN	*Linphonode status*
cT	*Tumor size*
DFS	Sobrevida livre de doença
DNA	Ácido desoxirribonucléico
dNTP	Desoxirribonucleotídeo Trifosfatado
dsDNA	DNA dupla-fita
DTT	Ditiotreitol
ELISA	Ensaio imunoenzimático
ER	Receptor de Estrógeno
Fab	Fragmento ligante de antígeno
Fc	Região constante
FDA	Administração de alimentos e drogas
Fv	Região variável
G	Gramas
G	Guanina
Gly	Glicina (aminoácido)
H	Hora
HA	Hemaglutinina
HCl	Ácido clorídrico

HER-2	Receptor 2 do Fator de crescimento epidermal humano
His	Histidina
H_2O	Água
HPLC	Cromatografia líquida de alta performance
HRP	Peroxidase
Ig	Imunoglobulinas
IG	Região Intergênica
IHC	Imunohistoquímica
INCA	Instituto Nacional do Câncer
INGEB	Instituto de Genética e Bioquímica
IPTG	Isopropil-β-D-thiogalactopiranosídeo
IUAC	União Internacional contra o Câncer
J	Segmento de Junção
KCl	Cloreto de Potássio
kDA	Quilodaltons
M	Molar
mAbs	Anticorpos monoclonais
µg	Micrograma
µL	Microlitros
mg	Miligramas
$MgCl_2$	Cloreto de Magnésio
Min	Minutos
mL	Mililitros
mM	Milimolar
mRNA	Ácido Ribonucléico Mensageiro
ng	Nanogramas
OS	Sobrevida global
pb	Pares de base
pComb3X	Vetor de clonagem
PCR	Reação em cadeia da polimerase
PD	*Phage Display*
PBS	Tampão Fosfato-Salino
PBST	Tampão Fosfato-Salino com Tween-20
pmoles	Picomoles
%	Porcentagem
PgR	Receptor de Progesterona

PIII	Proteína III do capsídio de bacteriófagos filamentosos
PVIII	Proteína VIII do capsídio de bacteriófagos filamentosos
RNA	Ácido Ribonucléico
RT	Transcrição reversa
scFv	Fragmento variável de cadeia única
Ser	Serina (aminoácido)
ssDNA	DNA simples-fita
T	Timina
Taq	*Thermus aquaticus* (Enzima DNA Polimerase)
TNBC	*Triple Negative Breast Cancer*
TNM	Tumor-linfonodo-metástase
U	Unidade de Atividade Enzima
UFU	Universidade Federal de Uberlândia
UTDL	Unidade Terminal Ducto-Lobular
VH	Domínio Variável de cadeia pesada
VL	Domínio Variável de cadeia leve

APRESENTAÇÃO

O Câncer de Mama (CM) é uma alteração genética herdada ou adquirida influenciada por fatores ambientais, comportamentais e reprodutivos. Sua incidência cresce a cada ano, o que o torna importante no cenário epidemiológico global. Trata-se de uma doença que engloba uma multiplicidade de entidades com características biológicas e comportamento clínico distintos, sustentados pela complexidade molecular que incluem alterações de vias e funções gênicas. Portanto, não apenas análises de expressão como também o estudo molecular desses tumores têm um profundo impacto na compreensão de seus mecanismos evolutivos e dos fatores responsáveis pela sua gênese, desenvolvimento e progressão

Tradicionalmente, a maioria dos métodos de identificação de marcadores tumorais é baseada na obtenção ou caracterização de anticorpos monoclonais capazes de reconhecer potencias alvos proteicos, no que diz respeito ao seu envolvimento em vias de sinalização ou controle transcricional. Durantes as últimas décadas, inúmeros esforços têm sido voltados para o uso da biologia molecular no desenvolvimento de métodos combinatoriais capazes de estabelecer uma conexão entre as proteínas e seu respectivo DNA codificante. Portanto, o progresso técnico na busca de ligantes e alvos específicos a tumores de mama tem sido acompanhado pelo conhecimento crescente de expressão e obtenção de possíveis alvos, sejam eles haptenos, proteínas ou ácidos nucléicos. Nesse sentido, técnicas como o *Phage Dislplay* oferecem ferramentas tanto para a evolução molecular quanto para a seleção por afinidade proteica.

O direcionamento terapêutico do Câncer de Mama exige esforços que incluam múltiplas abordagens, incluindo cirurgia, quimioterapia, radioterapia e hormonioterapia. Além disso, a busca de marcadores tumorais tanto para o diagnóstico quanto para o prognóstico é imprescindível para a detecção e o acompanhamento da evolução clínica da doença.

A engenharia de anticorpos tem permitido a geração de moléculas com características desejáveis e com significativo impacto nas pesquisas sobre a biologia de tumores. Nesse trabalho apresentamos, no Capítulo I, uma revisão de literatura a respeito dos aspectos clínico-patológicos do Câncer de Mama assim como seu perfil epidemiológico nos cenários mundial e brasileiro.

No Capítulo II apresentamos a seleção por *Phage Display* e a validação de um fragmento de anticorpo tipo Fab, denominado FabC-4 que, quando analisado quanto ao seu comportamento diante das características clínicas das pacientes com Câncer de Mama, apresentou valor diagnóstico e correlação com parâmetros prognósticos como grau histológico e classificação molecular. Considerando apenas o subtipo de tumores triplo-negativos, esse marcador, adotado como parâmetro de marcação, apresentou bom valor prognóstico, o que poderá, portanto, auxiliar na compreensão da heterogeneidade desse grupo de pacientes. Além disso, o anticorpo Fab-C4 foi capaz de reconhecer um epítopo conformacional da citoqueratina 10, a qual faz parte de um conjunto de filamentos intermediários responsáveis pelo controle de inúmeras atividades relacionadas ao surgimento e desenvolvimento de tumores como: controle tranducional e de proliferação celular.

Contudo, a imunogenicidade tumoral não se restringe a antígenos isolados, mas a múltiplos marcadores, envolvidos em inúmeros processos e cascatas de sinalização. Por esse motivo, no Capítulo III e IV buscamos definir o perfil transcricional dessas proteínas do citoesqueleto visando compreender seu papel, não somente como moléculas estruturais, como também sua importância nas cascatas de sinalização celular e em processos regulatórios.

CAPÍTULO 1

Fundamentação Teórica

RESUMO

O Câncer de Mama é uma doença heterogênea com diferentes sintomas ou sinais e características moleculares e comportamentos clínicos distintos. Após o câncer de pele não melanoma, o CM é o segundo mais comumente diagnosticado em mulheres no Brasil e nos Estados Unidos e a segunda causa de morte entre elas, após o câncer de pulmão.

Historicamente, a investigação de marcadores biológicos úteis na avaliação do CM tem priorizado aqueles presentes no sangue, tecidos-específcos, oncogenes, fatores de crescimento além de inúmeras proteínas de adesão, da matriz extracelular e do citoesqueleto. Nesse contexto, novas tecnologias têm ampliado as perspectivas diante da heterogeneidade dessa doença, abrindo caminhos para a evolução clínica na identificação de novas moléculas e vias de sinalização, imprescindíveis a sobrevivência de células neoplásicas. A revisão aqui apresentada visa descrever alguns aspectos associados à gênese e progressão do Câncer de Mama, explicitar o mecanismo de ação de anticorpos e seus fragmentos na busca de alvos clínicos, descrever a tecnologia de *Phage Display*, utilizada na construção e seleção de bibliotecas combinatoriais de anticorpos e abordar algumas implicações das citoqueratinas nessa doença, uma vez que são alvos moleculares do presente estudo.

Palavras chave: Câncer de Mama, Fab, *Phage Display*, citoqueratinas

1 EPIDEMIOLOGIA E ETIOLOGIA DO CÂNCER DE MAMA

O nome câncer abrange um vasto conjunto de doenças caracterizadas por apresentar um grupo de células que cresce aparentemente sem controle algum. O câncer está entre as primeiras causas de morte, ao lado das cardiopatias, doença do aparelho respiratório, doenças infecciosas e parasitárias (BOYLE e LEVIN, 2008).

A necessidade de medidas paliativas que diminuam o progresso tumoral e melhorem a qualidade de vida dos pacientes tem evidenciado a importância do câncer no cenário global de doenças em todo o mundo. Estima-se que o número de novos casos esperados aumentará de 20 milhões no ano 2000 para 30 milhões em 2020. Globalmente, aproximadamente 1,38 milhões de mulheres são diagnosticadas com CM e 458.503 morrem anualmente (LEE et al., 2012).

O Câncer de Mama (CM) vem atingindo progressivamente um número maior de mulheres, em faixas etárias mais baixas, com taxa de mortalidade também crescente no Brasil. Dados do INCA demonstraram que, entre 1979 e 1999, houve um aumento de 69% na taxa bruta de mortalidade por Câncer de Mama no Brasil (5,77 para 9,75 óbitos por 100 mil mulheres/ano). As estimativas para o ano de 2012 serão válidas também para o ano de 2013 e apontam a ocorrência de aproximadamente 52.680 casos novos desse câncer, com um risco estimado de 52 casos a cada 100 mil mulheres, reforçando a magnitude do problema do CM no país. O Rio de Janeiro apresenta a maior incidência para esse tipo de tumor (94,93/100.000). O estado de Minas Gerais tem uma taxa estimada de 45,04 casos para cada 100.000 mulheres (INCA, 2012).

O CM é uma doença heterogênea com diferentes sintomas ou sinais e características moleculares e comportamentos clínicos distintos. Sem considerar os tumores de pele não melanoma, é o segundo mais comumente diagnosticado em mulheres no Brasil e nos Estados Unidos e a segunda causa de morte entre elas, após o câncer de pulmão. A incidência anual estimada de CM em todo o mundo corresponde a um milhão de casos, com 200 mil casos nos Estados Unidos e 320 mil casos na Europa. Países como os Estados Unidos, Reino Unido, Suécia, Itália e Uruguai, apresentam taxas de incidência de CM superiores a 100 casos por 100 mil mulheres/ano. Consequentemente suas taxas de mortalidade

também são bastante elevadas, ficando ao redor de 40 óbitos por 100 mil mulheres/ano (DESANTIS et al., 2011).

Trata-se de uma alteração genética herdada ou adquirida influenciada por fatores ambientais, comportamentais e reprodutivos. Os principais fatores de risco para o desenvolvimento do CM estão relacionados a uma maior exposição do tecido mamário aos estrógenos circulantes. São fatores de risco estabelecidos a idade, menarca precoce, menopausa tardia, nuliparidade, primeira gestação tardia, obesidade na pós-menopausa, história familiar, hiperplasia atípica prévia e câncer na mama contralateral (MCPHERSON et al., 2000; MEISNER et al., 2008; GALVAO et al., 2011) (PETRACCI et al., 2011).

Alguns fatores de risco relacionados ao estilo de vida (álcool, dieta, obesidade e atividade física), densidade mamográfica, radiação ionizante, níveis de fator de crescimento semelhante à insulina (IGF-1) e prolactina, e fatores genéticos (genes de alta e baixa penetrância) também estão envolvidos com o surgimento da doença (DUMITRESCU e COTARLA, 2005). Contudo, apenas aproximadamente 10% dos casos de câncer de mama são familiares decorrentes da herança de mutações em genes de alta penetrância. Os genes supressores tumorais *BRCA1* e *BRCA2*, localizados nos braços longos dos cromossomos 17 e 13 respectivamente, são responsáveis por parcela importante dos casos de câncer de mama hereditários (MCPHERSON et al., 2000).

Apesar do desenvolvimento de novas técnicas cirúrgicas e dos avanços em tratamentos sistêmicos e radioterapia, observa-se que a mortalidade por essa afecção mantém-se em um patamar pouco variável, no entanto, quando detectado e tratado precocemente, as taxas de mortalidade se reduzem de forma significativa (GREEBAUM, 2000). O progresso científico e clínico para a detecção e tratamento da doença é evidente, uma vez que mulheres atualmente diagnosticadas com Câncer de Mama apresentam uma probabilidade de sobrevivência muito maior, comparadas às diagnosticas nos anos 70 e 80 (MEISNER et al., 2008). Portanto, devido à sua cura estar intimamente relacionada ao estádio de seu diagnóstico, muito necessita ser feito para um maior esclarecimento quanto à triagem e ao papel pessoal das pacientes na luta contra essa doença.

2 PATOLOGIA MOLECULAR DO CÂNCER DE MAMA

A glândula mamária é formada por um sistema de ductos ramificados a partir do mamilo, os quais se estendem radialmente entre o estroma fibrogorduroso. O complexo sistema de ductos ramificados pode ser dividido em dois grupos: a unidade terminal ducto-lobular (UTDL) e os grandes ductos (Figura 1). A UTDL é considerada a unidade anátomo-funcional da mama.

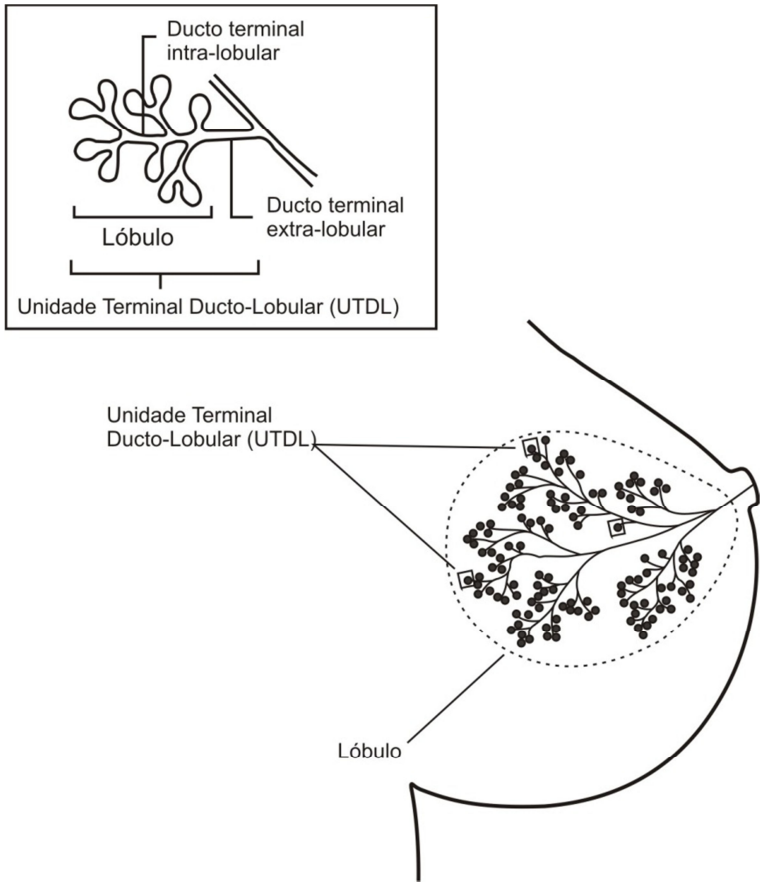

Figura 1: Micro-anatomia da mama. Fonte: REINIKAINEN, 2003.

As células epiteliais que compõem a glândula estão arranjadas em duas camadas: a camada epitelial luminal e a camada mioepitelial basal (Figura 2). Toda esta estrutura encontra-se circundada pela membrana basal (BRASILEIRO FILHO, 2006). A biologia e patologia da mama são embasadas nas células glandulares ou luminais e nas células mioepiteliais.

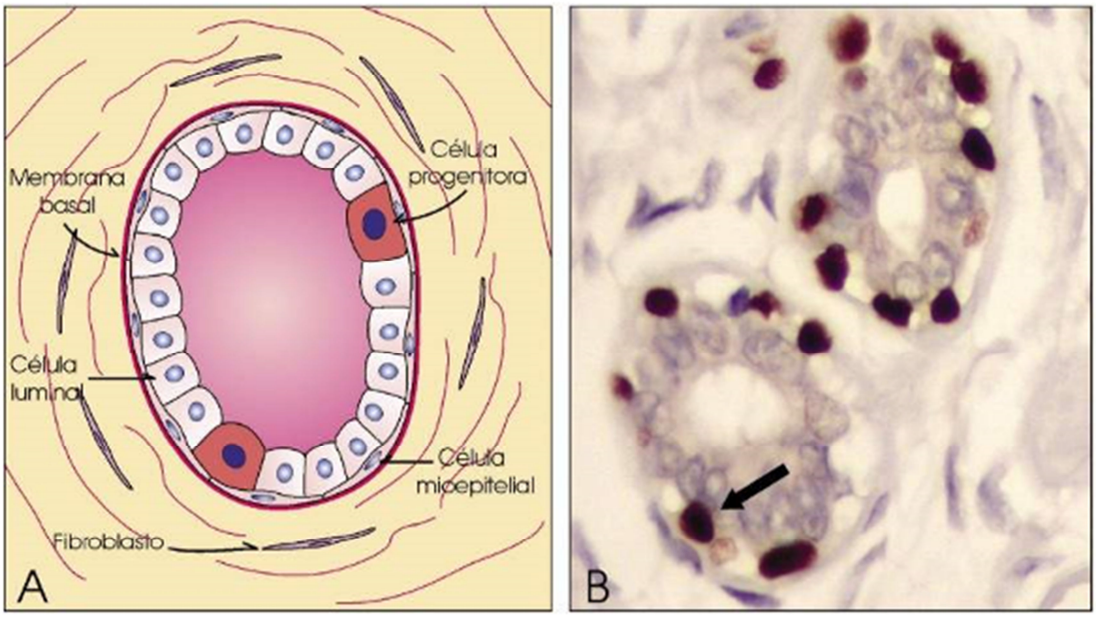

Figura 2: Composição celular da Glândula Mamária. **A.** Células epiteliais da glândula mamária. Representação do corte de um ducto mamário normal, no qual se observam células luminais, progenitoras e mioepiteliais. A membrana basal separa as células mioepiteliais do estroma adjacente. (Adaptada de Birnbaum, D *et al. Int. J. Oncol*., 25:249-58,2004.) **B.** Dúctulo de glândula mamária normal. Imunohistoquímica para uma proteína basal (p63) identifica os núcleos das células basais/mioepiteliais (seta). Fonte: Brasileiro Filho, G. *Bogliolo Patologia*. Sétima edição, Guanabara Koogan, 2006.

Os ductos e lóbulos são revestidos por uma camada luminal de células secretoras cuboidais. As células mioepiteliais estão em contato com a membrana basal contendo proteínas de músculo liso (BIRNBAUM et al., 2004; MAYROSE et al., 2007). No tecido mamário humano normal, os ductos e os lóbulos mamários estão delineados por duas camadas celulares distintas, uma superficial, formada por células epiteliais que estão em contato direto com a luz do ducto, denominada luminal, e outra interna que possui íntima relação com a membrana basal à qual está justaposta, denominada basal (GUSTERSON et al., 2005). Histologicamente, a maior parte dos cânceres esporádicos da mama tem origem nas células epiteliais luminais, sendo este fato apoiado por evidências morfológicas, bioquímicas e moleculares (CALLAGY et al., 2003).

O Câncer de Mama consiste em um conjunto de diferentes alterações que afetam o mesmo órgão e que se origina da mesma estrutura anatômica, mas que

possui diferentes fatores de risco, evolução clinica, características histopatológicas, resultados e resposta a terapia (REIS-FILHO e PUSZTAI, 2011). Em níveis biológicos, o CM é uma alteração extremamente complexa, originada por inúmeras mudanças genéticas e epigenéticas que mudam o curso de diferentes eventos incluindo proliferação celular, apoptose e angiogênese, com conseqüente aquisição de fenótipos malignos (GALVAO et al., 2011).

Na prática clínica, o diagnóstico histopatológico fornece elementos necessários para uma adequada classificação do estadiamento do câncer, apresentando a descrição das características da neoplasia, do estado linfonodal e do comprometimento das margens cirúrgicas de ressecção (SALLES et al., 2005). A Classificação Internacional de Doenças para Oncologia (CID-O) classificou os carcinomas da mama em: i) doença de Paget da aréola; ii) não-invasivos (ductal e lobular in situ) e iii) invasivos (ductal, lobular, mucinoso, medular, papilar, tubular, adenóide cístico, secretor juvenil, apócrino, escamoso, fusiforme, cartilaginoso e ósseo, tipo misto).

O estadiamento é realizado com base na classificação dos tumores malignos segundo seu tamanho, o comprometimento dos linfonodos e a ocorrência ou não de metástases (TNM), proposta pela *International Union Against Cancer* (IUAC). O tamanho do tumor juntamente com a condição dos linfonodos axilares são dois importantes indicadores prognósticos para o câncer de mama, pois auxiliam de forma significativa o direcionamento clínico (GEYER et al., 2009).

O CM tem sido o tumor epitelial maligno mais amplamente estudado por métodos moleculares o que permitiu uma caracterização sistemática de seus subtipos, os quais ampliaram o conhecimento prognóstico e preditivo da doença (LEONG e ZHUANG, 2011). Análises de expressão utilizando microarranjos de nucleotídeos têm demonstrado que o CM pode ser classificado em pelo menos cinco grupos: Luminal A, Luminal B, Her-2, Basal e Tumores molecularmente semelhantes à mama normal (Figura 3). Recentemente, um novo subtipo identificado como Claudina-*low*, foi acrescentado ao grupo (PEROU et al., 2000; SORLIE et al., 2001; MALHOTRA et al., 2010).

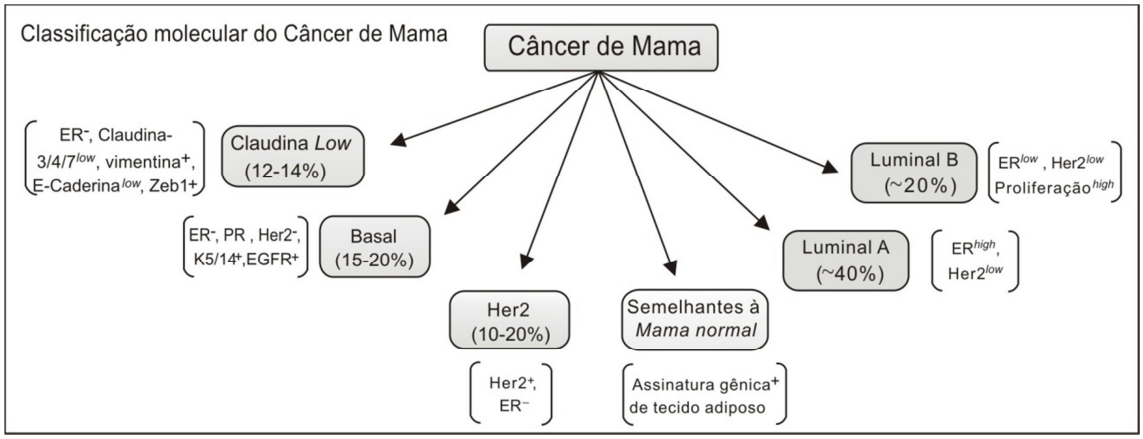

Figura 3: Classificação Molecular dos tumores de mama. Fonte: Adaptado de (MALHOTRA et al., 2010)

A principal diferença observada entre os subtipos moleculares se refere à expressão do Receptor de Estrógeno (ER). Os tumores luminais são descritos como aqueles que apresentam um padrão de expressão reminiscente de células epiteliais luminais normais, incluindo a presença de citoqueratinas (CK) de baixo peso molecular (8/18), ER e genes associados com a via desse hormônio (SORLIE et al., 2003; BRENTON et al., 2005). Quanto aos subgrupos, aqueles classificados como Luminal A apresentam, em sua maioria, baixo grau histológico, excelente prognóstico e maior expressão de genes relacionados a ER. Já os Luminal B apresentam elevado grau histológico, maior capacidade proliferativa e um pior prognóstico. Os tumores molecularmente semelhantes à mama normal são pouco caracterizados e sua significância clínica ainda necessita ser estabelecida. Os subtipos Her-2 são, de um modo geral, negativos para ER e caracterizados pela superexpressão de Her-2, de genes associados à via EGFR/HER2 (PIGNOCHINO et al., 2010) e/ou de sua amplificação no cromossomo 17q22. Apresentam comportamento clínico agressivo, mas passíveis de tratamento com drogas anti-Her2 (GEYER et al., 2009).

Os tumores basais de mama são definidos como aqueles com características basais/mioepiteliais, com elevado grau nuclear, elevada atividade mitótica, co-expressão de citoqueratinas de alto peso molecular (CK5/6/14/17) e vimentina, e negativos para ER e Receptor de Progesterona (PR) (GEYER et al., 2009). Além disso, esses tumores podem apresentar mutações no gene *TP53* e

pacientes portadores da mutação *BRCA1* frequentemente são classificados nesse subgrupo.

A aplicabilidade da metodologia de microarranjos de cDNA permitiu a identificação de subtipos basais e sua correlação com as características do tumor e perfil clinico das pacientes (CHOI et al., 2010; RATKAJ et al., 2010). Contudo, características morfológicas são amplamente associadas a alterações na mama, definindo, inclusive, importantes fatores preditivos no CM. Nesse sentido, tumores classificados como Triplo-Negativos são definidos, por imunohistoquímica, como aqueles que não possuem expressão dos receptores hormonais (estrógeno e progesterona) e Her2, singularmente interessantes pela ausência de terapia adjuvante (FOULKES et al., 2010).

Embora o fenótipo basal se correlacione fortemente com a ausência de expressão de ER, PR e Her-2, também conhecido como Tumores de Mama Triplo Negativos (TMTN), não se tratam da mesma entidade. Nem todos os TMTN são basais, e a presença de marcadores basais CK5/6 e/ou EGFR têm identificado um comportamento clínico agressivo por parte desses pacientes, com maiores chances de recorrência nos primeiros cinco anos (MALHOTRA et al., 2010; LEONG e ZHUANG, 2011; AMOS et al., 2012).

Portanto, o CM engloba uma multiplicidade de entidades com características biológicas e comportamento clínico distintos, sustentados pela complexidade molecular que engloba alterações de vias e funções gênicas. As análises de expressão associadas ao estudo molecular desses tumores têm um profundo impacto na compreensão de seus mecanismos evolutivos e dos fatores responsáveis pela sua gênese, desenvolvimento e progressão (GEYER et al., 2009).

Historicamente, a investigação de marcadores biológicos úteis na avaliação do CM tem priorizado aqueles presentes no sangue, tecido-específicos, oncogenes, fatores de crescimento além de inúmeras proteínas de adesão, da matriz extracelular e do citoesqueleto (GRUVER et al., 2011). Nesse contexto, novas tecnologias têm ampliado as perspectivas diante da heterogeneidade dessa doença, oferecendo novas perspectivas clínicas na identificação de novas moléculas e vias de sinalização, imprescindíveis à sobrevivência de células neoplásicas (LEONG e ZHUANG, 2011; AMOS et al., 2012).

3 MAPEAMENTO DAS INTERAÇÕES PROTEÍNA-PROTEÍNA POR MÉTODOS BIOLÓGICOS COMBINATORIAIS

A constante busca por novos alvos terapêuticos capazes de reconhecer a diversidade molecular do câncer requer estratégias específicas de identificação de marcadores que possuam relevância clínica (SERGEEVA et al., 2006). O estudo das interações proteína-proteína pode auxiliar na determinação das funções biológicas desses alvos e de seus papéis nos diversos mecanismos celulares (WALHOUT e VIDAL, 2001).

Uma estratégia ideal para a detecção de interações entre proteínas deve excluir resultados insatisfatórios e ser capaz de permitir a análise de múltiplas sequências obtidas. Durante as últimas décadas, inúmeros esforços têm sido voltados para o uso da biologia molecular no desenvolvimento de métodos combinatoriais capazes de estabelecer uma conexão entre as proteínas e seu respectivo DNA codificante (PELLETIER e SIDHU, 2001).

Tradicionalmente, a maioria dos métodos de identificação de marcadores tumorais é baseada em anticorpos monoclonais contra proteínas das quais se tenha alguma suspeita e, dessa forma, buscam os mesmos marcadores para diversos tipos de cânceres. A utilização de metodologias que fazem uma varredura das células tumorais, sem conhecimento prévio das proteínas nelas presentes, propicia a identificação de novos marcadores. A técnica de bibliotecas apresentadas na superfície de fagos permite a utilização não apenas de um anticorpo monoclonal, mas de uma vasta biblioteca de anticorpos ou peptídeos contra o conjunto das proteínas do tumor (AUSTIN, 1989). Inicialmente desenvolvida para o mapeamento de epítopos, essa técnica tem se tornado uma ferramenta poderosa para identificação de alvos protéicos com aplicações em pesquisa básica e aplicada (BARBAS, C. F. et al., 2001)

O Phage Display (PD) é o método combinatorial predominante que se baseia na expressão de peptídeos ou proteínas no exterior da partícula viral, enquanto o material genético codificante permanece em seu genoma (SMITH e PETRENKO, 1997; AZZAZY, H. M. e HIGHSMITH, W. E., JR., 2002). Utilizando as técnicas de DNA recombinante, coleções de bilhões de peptídeos, variantes protéicos ou produtos de fragmentos gênicos (ou cDNA) podem ser apresentados

na superfície de bacteriófagos e submetidos a inúmeras estratégias de seleção (BRATKOVIC, 2010).

Os fagos recombinantes expressando peptídeos randômicos podem ser selecionados por afinidade e a seguir expandidos em ciclos adicionais de crescimento em bactérias *E. coli* hospedeiras apropriadas (SMITH, 1985). A técnica de PD é baseada no uso de um bacteriófago filamentoso M13 capaz de infectar bactérias *E. coli* gram negativas. Os fagos filamentosos são ideais como veículos de clonagem e de expressão. Seu genoma é pequeno e tolera a inserção de sequencias em regiões não essenciais, a clonagem e construção da biblioteca são facilitadas pela habilidade de isolamento do material genético como DNA fita simple (ssDNA) e fita dupla (dsDNA). Além disso, podem ser utilizados vetores baseados em plasmideos, as proteínas do capsídeos podem ser modificadas sem comprometimento da atividade viral, apresentam ciclo lisogênico e as partículas virais são resistentes a uma ampla variedade de condições de seleção (RUSSEL et al., 2004).

A partícula de fago é formada por uma fita simples de DNA envolta por uma capa protéica constituída por cinco proteínas: pIII, pVI, pVII, pVIII e pIX conforme ilustrado na Figura 4 (RUSSEL et al., 2004). A técnica utiliza o princípio de que polipeptídeos podem ser expressos na superfície desses bacteriófagos pela inserção de um segmento de DNA codificante no genoma dos mesmos, de modo que a proteína ou o peptídeo expresso fique exposto na superfície da partícula viral fusionado a uma proteína endógena, pIII ou pVIII (BARBAS, C. F. et al., 2001; BARBAS, C. F. et al., 2001) , enquanto seu material genético permanece no interior do fago (BENHAR, 2001). O fago M13, mais amplamente utilizado na técnica de PD, possui a capacidade de infectar *E. coli*, pela ligação da pIII ao *pilus* F da célula bacteriana (AZZAZY, H. M. e HIGHSMITH, W. E. J. R., 2002), as quais são utilizadas para recuperar os fagos ligados, montados no espaço periplasmático das células hospedeiras (BENHAR, 2001).

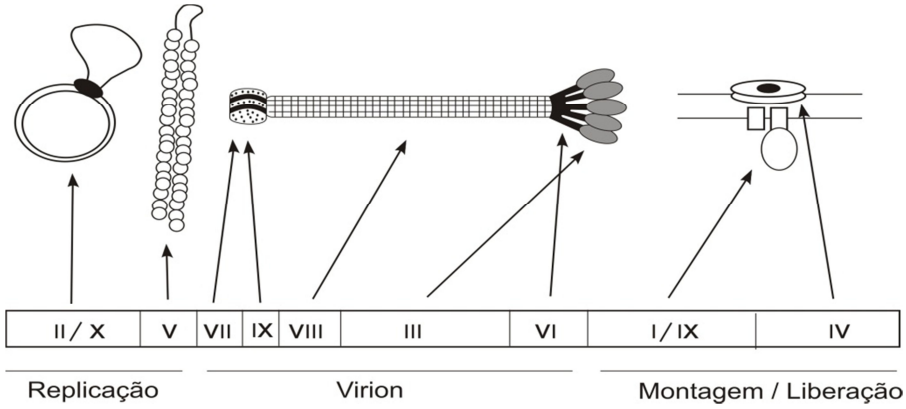

Figura 4: Representação dos genes e produtos gênicos do fago filamentoso f1 (M13/fd). *Gene II* codifica para a pII, a qual se liga na região intergênica (IG) do dsDNA identificando a fita + para o início da replicação do fago por proteínas da célula hospedeira. A pX é recrutada em um momento tardio da infecção, para a conversão do material genético em ssDNA. *Gene V* codifica para a proteína V ligante de ssDNA. *Genes VII e IX* codificam para duas proteínas pequenas localizadas na extremidade do vírus que primeiramente emerge da célula hospedeira durante a montagem do fago. *Gene VIII* codifica para a maior proteína do capsídeo viral e os *genes III e VI* codificam para as proteínas pIII e pVI, localizadas na porção final do vírus responsáveis por mediar o término da montagem e liberação do *virion* e o processo de infecção. *Gene I* codifica para duas proteínas transmembrânicas pI e pXI, e finalmente o *gene IV* que codifica para a pIV, um canal multimérico através do qual o fago é liberado sem que ocorra a lise celular. Fonte: (RUSSEL et al., 2004).

Há dois formatos básicos de expressão de polipeptídeos em bibliotecas de fagos: polivalente e monovalente. No formato polivalente, cada cópia da proteína pIII do capsídeo viral possui um polipeptídeo fusionado. Bibliotecas nesse formato são baseadas em vetores derivados diretamente do genoma do fago que codificam todas as proteínas necessárias para sua replicação e montagem (KEHOE e KAY, 2005). Os sistemas polivalentes são comumente usados em seleções a partir de bibliotecas de pequenos peptídeos, os quais não interferem nas funções desempenhadas pela pIII do vírus (SERGEEVA et al., 2006). O formato monovalente é o sistema usado no caso de bibliotecas de cDNA, os quais codificam proteínas ou domínios que, devido ao seu tamanho, comprometem o funcionamento da pIII. Portanto, um mosaico de proteínas recombinantes e selvagens é produzido a partir de um vetor do tipo fagomídeo (que codifica a proteína fusionada) e por um fago *helper*, respectivamente. Nesse sistema, o fago *helper* é responsável por providenciar todas as proteínas necessárias para a

replicação, produção do ssDNA, montagem e liberação de partículas virais (SERGEEVA et al., 2006). Durante a infecção viral, o DNA proveniente dos fagomídeos é preferencialmente revestido pelas proteínas estruturais, pois os fagos *helper* possuem mutações na origem de replicação, dificultando a reprodução e empacotamento de seu próprio material genético (BARBAS, C. F. et al., 2001). Na figura 5 estão ilustradas as duas alternativas de clonagem e expressão de peptídeos para seleção por PD.

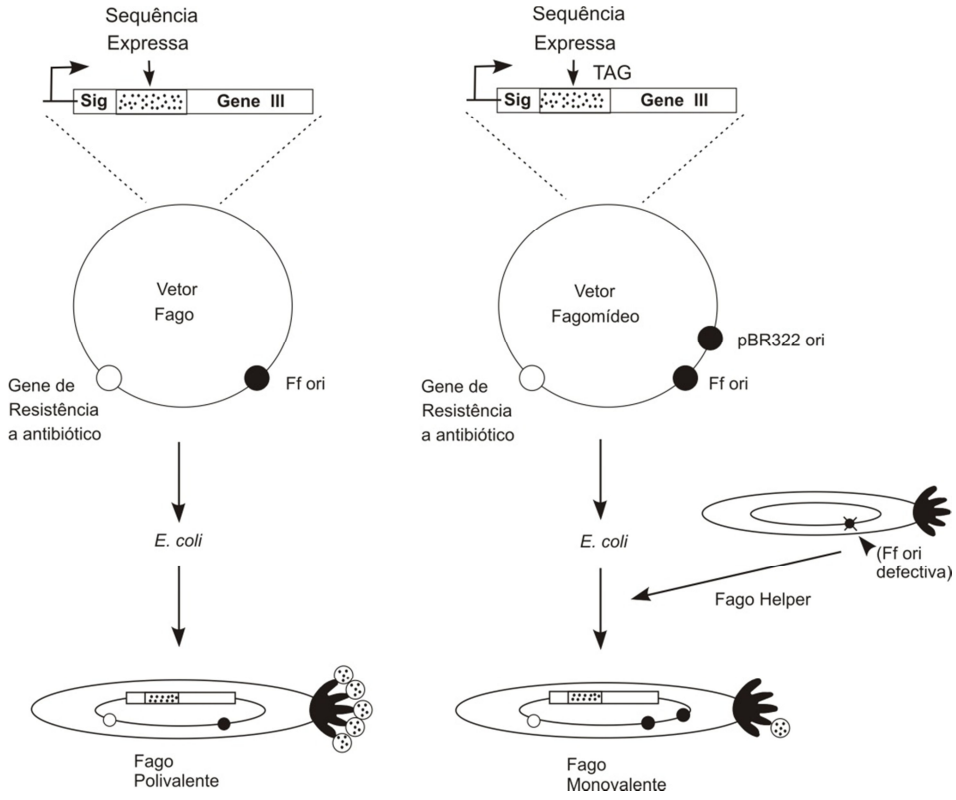

Figura 5: Esquema geral para PD usando como vetores fagos e fagomídeos. As diferenças estão ilustradas para proteínas fusionadas à pIII. As sequencias são inseridas entre o peptídeo sinal e o gene III. Ambos os sistemas possuem a origem de replicação em fago (Ff) para a produção dos *virions*. Contudo, vetores do tipo fagomídeo também possuem origem de replicação de plasmídeo e um gene de resistência a antibióticos para sua seleção em *E.coli*. Em muitos fagomídeos um stop códon (TAG) é interposto entre a sequencia expressa e o gene III para a obtenção de partículas solúveis a partir da transformação do vetor em cepas não-supressoras de *E.coli*. Fonte: (RUSSEL et al., 2004).

Os polipeptídeos expressos na superfície de fagos possibilitam a seleção de seqüências baseadas em sua afinidade de ligação a uma molécula alvo (antígeno) por um processo de seleção por afinidade *in vitro*. Durante esse processo clones específicos são reamplificados e lavagens sucessivas garantem a remoção de fagos não ligantes (BARBAS, C. F. et al., 2001).

Os fagos resultantes deste processo são titulados e submetidos a um novo *biopanning* (ligação ao alvo, eluição e amplificação) visando o enriquecimento das sequências específicas para o alvo. Após três ou quatro repetições deste processo, clones individuais são submetidos a ensaios imunológicos e suas sequências de DNA podem ser obtidas por sequenciamento (BARBAS, C. F. et al., 2001).

As doenças humanas podem ser causadas e/ou estar associadas com alterações na expressão protéica. A evidente heterogeneidade sujeito-específica corrobora essa necessidade por metodologias que busquem perfis individuais de marcadores. A identificação de alvos confiáveis permitirá o desenho de novas terapias assim como avanços na predição clínica de tecidos patologicamente afetados. Esses objetivos são particularmente importantes ao se considerar doenças malignas, como o câncer (SERGEEVA et al., 2006).

A tecnologia de PD tem sido uma ferramenta promissora na identificação e validação de alvos protéicos, seja na construção e busca de anticorpos recombinantes ou na seleção de seus prováveis antígenos. A identificação de novos marcadores ou prováveis vias de sinalização pode permitir o desenho de novas estratégias diagnósticas e terapêuticas, assim como predizer a evolução clínica de tecidos afetados por doenças heterogêneas. Nesse contexto, o PD tem se destacado em suas aplicações em imunologia, engenharia de anticorpos e estudos de interações protéicas (DE BRUIN et al., 1999).

4 ANTICORPOS: ASPECTOS ESTRUTURAIS E PRINCÍPIOS BIOTECNOLÓGICOS

A molécula de anticorpo é baseada em um estrutura de quatro cadeias, que compreendem duas cadeias pesadas idênticas (50kDa) e duas cadeias leves idênticas (25kDa), organizadas em três diferentes unidades (Figura 6). Duas dessas unidades permitem o reconhecimento antigênico, sendo conhecidas como

porção Fab (fragmento de ligação ao antígeno) da molécula. A terceira unidade, Fc (fragmento constante) encontra-se geralmente envolvida com o recrutamento de funções efetoras, como ativação do sistema complemento (BARBAS, C. F. et al., 2001; KIM et al., 2005).

Existem cinco classes de anticorpos ou imunoglobulinas humanos (Ig): IgM, IgG, IgE, IgA, e IgD as quais são altamente reativas a alvos específicos, sendo assim responsáveis pela principal defesa contra organismos patogênicos e toxinas (HOLLIGER e HUDSON, 2005). As IgGs são as imunoglobulinas mais abundantes no sangue humano (85% das Igs séricas) e as mais amplamente utilizadas para diagnóstico e terapia (WEISSER e HALL, 2009). O sítio de ligação ao antígeno é formado por seis regiões determinantes de complementariedade (CDR). Ambas as regiões hipervariáveis das cadeias leve (VL) e pesada (VH) possuem três CDRs, cuja variabilidade é garantida por rearranjos gênicos, recombinação somática e hipermutação somática, gerando diferentes sítios de ligação aos antígenos em linfócitos B (FILPULA, 2007; ROMER et al., 2011).

Interessantemente, o evento de hipermutação somática pode ser reproduzido em laboratório, sendo a tecnologia de *Phage Display* uma dessas ferramentas (CLEMENTI et al., 2012). Os múltiplos ciclos de crescimento associados a uma elevada taxa de proliferação e estringência de seleção, favorecem o isolamento de mutantes com maior capacidade de reconhecimento do antígeno. Portanto, a presença de mutações nos clones isolados reproduz, *in vitro*, o fenômeno de variabilidade identificado nos eventos de hipermutação, o que possibilita a utilização da técnica na geração de biblioteca de anticorpos com elevada variabilidade e capacidade de reconhecimento antigênico (LOW et al., 1996).

Os domínios funcionais e estruturais dos anticorpos podem ser separados por digestão protéica. A papaína, por exemplo, cliva a molécula de IgG na região de dobradiça e libera três fragmentos: dois Fab (fragmento de ligação ao antígeno) e um Fc (fragmentos constantes). Já a pepsina degrada o fragmento Fc e libera dois Fab ligados $(Fab)_2$. Essa característica se torna particularmente importante, pois inúmeras aplicações dessas moléculas não requerem a função efetora da porção Fc, como a longa meia-vida sérica em imagens com contraste e a inapropriada ativação e liberação massiva de citocinas além de efeitos tóxicos

associados. As características estruturais de uma molécula de anticorpo encontram-se representadas na Figura 5:

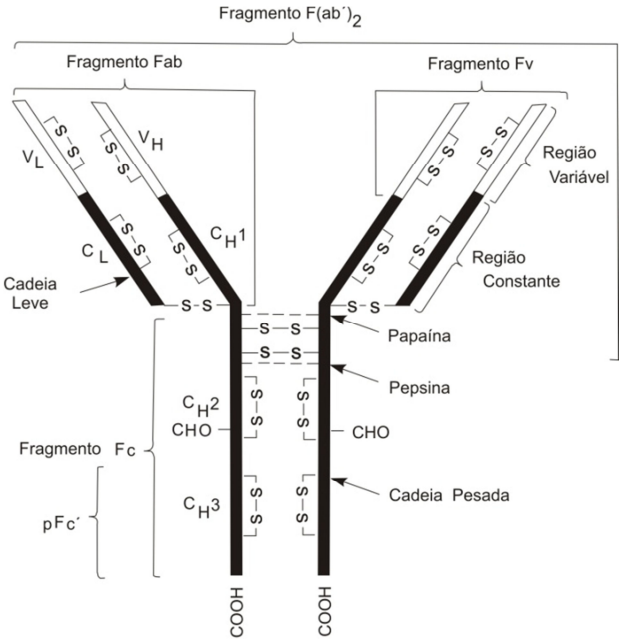

Figura 6: Representação das quatro cadeias estruturais de uma IgG. Pontes dissulfeto (S-S) ligam as duas cadeias H e também as cadeias L e H. Os fragmentos gerados por clivagem proteolítica estão representados pelas setas. H: cadeia pesada; L: cadeia leve. Fonte: (BARBAS, C. F. et al., 2001).

Com o desenvolvimento das técnicas de DNA recombinante criou-se a possibilidade de produzir fragmentos funcionais de anticorpos baseados na manipulação das suas seqüências codificadoras, o que possibilita gerar diversas combinações funcionais com grande potencial clínico (AZZAZY, H. M. e HIGHSMITH, W. E., JR., 2002; HOLLIGER e HUDSON, 2005).

A principal tecnologia desenvolvida para a produção de anticorpos humanos foi realizada em camundongos transgênicos que expressavam um repertório de sequencias gênicas dessas moléculas (LONBERG, 2005). Embora essa abordagem tenha gerado anticorpos monoclonais (mAbs) humanos de alta afinidade, alternativas *in vitro* para a construção e seleção de bibliotecas de anticorpos, via expressão viral ou celular, podem proporcionar melhor rendimento e uma capacidade ótima de busca por ligantes específicos (WARK e HUDSON, 2006). Esses avanços exploram o funcionamento ribossomal ou a biologia

molecular de vetores, permitindo a manipulação gênica e o controle de seus produtos oferecendo a vantagem crucial de uma conexão direta entre o fenótipo experimental e o genótipo, o que possibilita uma evolução dos ligantes selecionados. Nesse contexto, o *Phage Display* tem sido amplamente usado para a geração de bibliotecas de fragmentos de anticorpos Fab ou scFv (fragmento variável de cadeia simples) (FILPULA, 2007; DANTAS-BARBOSA et al., 2012).

Os fragmentos do tipo Fab consistem em segmentos VH-CH e VL-CL, unidos por pontes dissulfeto. O fragmento menor Fv (fragmento variável) é composto apenas pelas regiões VL e VH. A versão recombinante desse fragmento é conhecida como scFv. Nesse formato, as duas regiões variáveis são artificialmente unidas por um peptídeo flexível (adaptador) composto por 15 aminoácidos de sequencia (Gly4Ser)3 e expressas como uma cadeia polipeptídica simples. Na Tabela 1 encontram-se resumidas algumas características de diferentes fragmentos de anticorpos (AZZAZY, H. M. e HIGHSMITH, W. E., JR., 2002).

Tabela 1: Características de fragmentos de anticorpos

Fragmento de Anticorpo	Tamanho (kDa)	Paratopos (Valência)	Estrutura
scFv	25-30	1	Domínios VH e VL são unidos por um *linker* de 15 aminoácidos. Mudanças no tamanho do *linker* direcionam a formação de *diabodies* (60kDa), *triabodies* (90kDa) ou *tetrabodies* (120kDa)
Fv	25	1	VH e VL sem *linker* entre os domínios variáveis
Minibody	80	2	scFv-CH3 em um dímero bivalente de 80kDa
Fab	50	1	Composto por duas cadeias: VH-CH e VL-CL
$F(ab')^2$	100	2	Duas moléculas Fab

Fonte: Adaptado de (AZZAZY, H. M. e HIGHSMITH, W. E., JR., 2002)

Desde 2008, a engenharia de anticorpos tem conquistado cerca de 30% do campo biotecnológico. Esses dados demonstram que a construção de fragmentos de anticorpos tem apresentado, progressivamente, um importante papel na busca de biomarcadores e no tratamento do câncer, ao identificar antígenos tumorais. O desenvolvimento de métodos de expressão dessas moléculas em bactérias e sua

apresentação no capsídeo de fagos filamentosos têm se mostrado como uma tecnologia robusta capaz de gerar anticorpos recombinantes, mimetizando o próprio sistema imune na seleção clonal (KIPRIYANOV e LE GALL, 2004; KIM et al., 2005).

O fragmentos de anticorpos do tipo Fab são, de fato, os mais amplamente explorados. Todo o conhecimento e experiência científicos foram gerados durante o desenvolvimento e aprovação, pela FDA (*Food and Drug Administration* – EUA) de três drogas para uso terapêutico (Abciximab, Ranibizumab, Certolizumab pegol); seis agentes em desenvolvimento clínico ativo e 20 programas descontinuados, os quais coletivamente são responsáveis por 49% dos 54 fragmentos de anticorpos identificados (NELSON, 2010).

O formato Fab permite uma rápida seleção entre um grande número de clones, uma vez que são fragmentos cuja formação é predominantemente monomérica (DE HAARD et al., 1999). Além disso, são mais estáveis e propícios de reterem sua estrutura natural e capacidade de reconhecimento e afinidade ao seu alvo, sem problemas quanto à avidez observada em bibliotecas multiméricas de scFv (ZHANG et al., 2007).

Para a construção de uma biblioteca Fab os repertórios gênicos das porções variável e constante do anticorpo podem ser amplificados a partir da transcrição reversa do mRNA extraído do sangue total de pacientes. Os oligonucleotídeos iniciadores são capazes de cobrir todas as famílias gênicas originando, assim, anticorpos humanos por engenharia genética. A biblioteca é gerada pela combinação randômica das regiões variáveis das cadeias leve e pesada produzindo fragmentos Fab capazes de reconhecer um antígeno específico. O fragmento de DNA correspondente ao Fab é então inserido no vetor fagomídeo pcomb3XSS, transformada em bactéria *E.coli* competente que, por sua vez, é infectada por partículas virais (BARBAS, C. F. et al., 2001)..

O fagomídeo pcomb3XSS (Figura 7) é um vetor que apresenta um único promotor *lac* e duas sequencias *leader ompA* e *pelB* responsáveis por direcionarem a expressão da cadeia leve e da cadeia pesada fusionada à pIII; respectivamente. O gene III desse vetor possui de 230 a 406 aminoácidos e a clonagem direcional é garantida pelos sítios de restrição da enzima *SfiI*. Essa enzima reconhece oito pares de bases da sequencia GGCCNNNN^NGGCC, cliva

na sua porção degenerada e, portanto, apresenta sítio de restrição assimétrico. A presença dos sítios únicos 5' (GGCCCAGG^CGGCC) e 3' (GGCCAGGC^CGGCC) permite uma correta orientação durante a ligação e, portanto, facilita a construção de bibliotecas combinatoriais complexas. Sítios dessa enzima não são encontrados em imunoglobulinas e são extremamente raros na maioria dos genes. Além dessas características, esse vetor ainda apresenta dois peptídeos na região carboxiterminal da proteína para seu isolamento e detecção: uma região de seis histidinas (His_6) para a purificação em colunas de cromatografia e o epítopo Hemaglutinina (HA – YPYDVPDYAS), utilizado na imunodetecção a partir de anticorpos comerciais anti-HA. A presença de um códon âmbar TAG permite a produção solúvel da proteína clonada em linhagens não supressoras, sem a presença do gene III (BARBAS, C. F. et al., 2001).

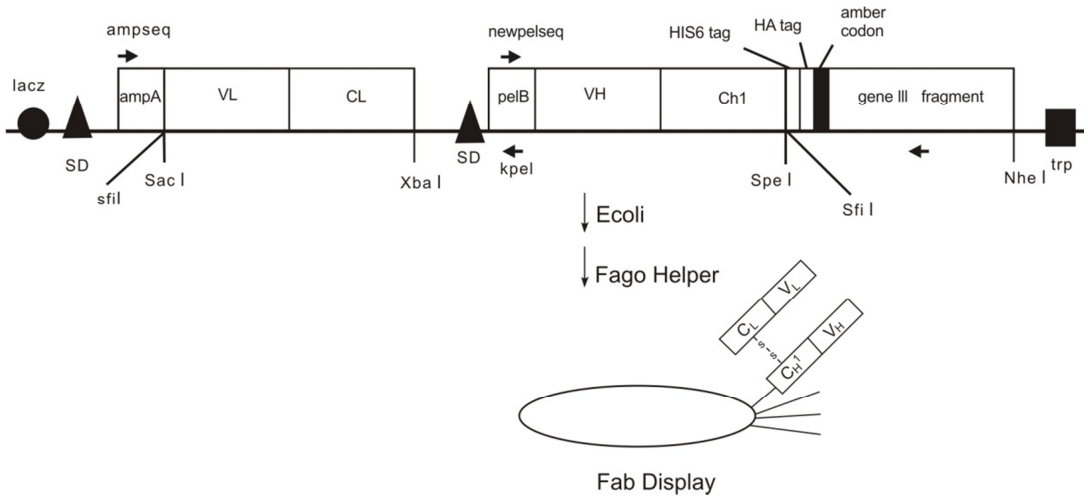

Figura 7: Representação esquemática do vetor pcomb3XSS. Fonte: (BARBAS, C. F. et al., 2001)

Para a produção solúvel de moléculas de Fab, linhagens não supressoras de *E.coli* são mais amplamente utilizadas do que sistemas eucarióticos, pois conduzem um maior rendimento, são de fácil manipulação e possuem baixo custo. Além disso, o amplo conhecimento do funcionamento molecular desses organismos facilita a manipulação e caracterização da proteína expressa (LEONG e CHEN, 2008). A expressão do anticorpo é regulada por promotores induzíveis como o promotor *lac* com a presença do indutor IPTG (Isopropil-β-D-

tiogalactosídeo) (WEISSER e HALL, 2009). Contudo, essa produção pode se tornar tóxica para a célula hospedeira. Portanto, é necessário um controle de todo o processo que pode ser realizado a partir de uma repressão catabólica, com a adição de glicose (O'BRIEN e AITKEN, 2003). Por outro lado, esse vetor pode oferecer anticorpos solúveis secretados diretamente no espaço periplasmático, uma vez que esse ambiente contribui para a correta formação de pontes dissulfeto entre os domínios da molécula (AHMAD et al., 2012).

Em trabalho anterior, nós (ARAÚJO, 2009) construímos uma biblioteca no formato Fab a partir do sangue periférico de pacientes diagnosticadas com câncer de mama. A partir dessa biblioteca, foi selecionado o clone Fab-C4, o qual apresentou reatividade diferencial entre proteínas extraídas de tecidos com Câncer de Mama, Tumor benigno e Normal. Após indução, moléculas solúveis de Fab-C4 foram capazes de reconhecer antígenos em lâminas de pacientes, novamente caracterizando os grupos de estudo. Contudo, ainda não foram elucidados os papéis clínicos e terapêuticos desse fragmento.

De fato, a habilidade de anticorpos ou fragmentos de anticorpos de se ligarem com elevada afinidade e especificidade a alvos moleculares prevalentes em uma determinada doença, mas não em células normais, tem sido o fundamento para a escolha de promissores ligantes antigênicos. Felizmente, a revolução tecnológica na engenharia de anticorpos ocorreu concomitante à expansão do conhecimento da biologia e patologia celular em níveis moleculares acompanhados pelo progresso nas áreas genômica e proteômica, abrindo novas fronteiras na prática clínica (FILPULA, 2007).

5 FAB: APLICAÇÕES CLÍNICAS

Comparados às moléculas de imunoglobulinas, os fragmentos de anticorpos apresentam inúmeras vantagens nos ensaios clínicos o que incluem maior penetração no tumor, clareamento sanguíneo mais rápido, menor tempo de retenção inespecífica e reduzida imunogenicidade. Apesar e um formato reduzido, fragmentos do tipo Fab são capazes de reter a capacidade de ligarem ao antígeno, mantendo sua aplicabilidade na área oncológica (DE MARCO, 2011; AHMAD et al., 2012).

O crescente número de anticorpos presentes nos ensaios clínicos são moléculas humanas derivadas da tecnologia de *Phage Display* ou de camundongos transgênicos capazes de expressar o repertório gênico das imunoglobulinas. Trata-se de tecnologias que oferecem caminhos robustos na geração de anticorpos humanos (CARTER, 2006).

Na medicina moderna, anticorpos com aplicação terapêutica têm se estabelecido como uma importante classe de drogas. As vendas mundiais dessas moléculas para fins diagnósticos/terapêuticos têm crescido nos últimos anos subindo de 26 bilhões de dólares em 2006 para mais de 30 bilhões de dólares em 2008, com uma projeção de 56 bilhões de dólares em 2012 (JEONG et al., 2011).

Atualmente, existe uma série de anticorpos e fragmentos de anticorpos aprovados (ou em testes) pelo FDA para uso terapêutico humano, sendo que a maioria destinada às aplicações clínicas tem origem na engenharia genética. Como exemplos, estão descritas moléculas Fabs, scFvs, anticorpos quiméricos e ainda os denominados humanizados (*human-like*) (Tabela 2) (CO e QUEEN, 1991; HOLLIGER e HUDSON, 2005). Contudo, a imunoterapia tem sido mais bem sucedida contra células neoplásicas circulantes, quando comparada a tumores sólidos (AHMAD et al., 2012).

Tabela 2: Fragmentos de anticorpos Fab e scFv aprovados (ou em testes) nos EUA para uso terapêutico.

Tipo de fragmento/ fonte	Nome (genérico)	Molécula alvo	Indicação
Fab/ camundongo	CEA-scan (arcitumomab)	CEA	Câncer colorretal
Fab/ humanizado	Thromboview	D-dímero	Trombose venosa
Fab/ humanizado	CDP791	VEGF	Anti-angiogenesise
Fab/ humanizado	CDP870	TNF-α	Doença de Crohn
Fab/ humanizado	MDX-H210	Her2/Neu e CD64 (FcR1)	Câncer de mama
Diabody $(V_H\text{-}V_L)_2$ /humano	C6.5K-A	Her2/Neu	Cânceres de mama e de ovário
Minibody quimérico	10H8	Her2	Cânceres de mama e de ovário
scFv / humano	F5 scFv-PEG	Her2	Câncer de mama
Minibody quimérico	10H8	Her2	Cânceres de mama e de ovário

Adaptado de Holliger & Hudson (2005).

Os principais modos de ação dos anticorpos na inibição do desenvolvimento de tumores incluem mecanismos imunes e mecanismos que interceptam as vias da tumorigênese. Dentre os mecanismos imunes encontram-se a ativação do sistema complemento e a resposta celular aos antígenos identificados. Os mecanismos que interceptam a tumorigênese abrangem tanto a indução apoptótica e o bloqueio angiogênico, quanto à inibição da proliferação celular com conseqüente interferência em inúmeras cascatas de sinalização e aceleração da internalização de receptores (CARTER, 2006; BEN-KASUS et al., 2007).

Os linfócitos B são reconhecidamente envolvidos na luta contra o desenvolvimento da doença, devido à sua resposta imunológica via secreção de imunoglobulinas antígeno-específicas. Apesar de estar claro o papel da ativação aguda desses linfócitos na erradicação de células neoplásicas recém formadas e na regressão da doença através de mecanismos envolvendo a biologia de anticorpos, estudos prévios têm indicado que a ativação crônica de células B pode, paradoxalmente, potencializar o desenvolvimento do carcinoma (CURIGLIANO et al., 2007; DENARDO e COUSSENS, 2007). Assim, fragmentos de anticorpos no formato Fab podem inibir essas funções efetoras, culminando em uma estratégia com propriedades superiores para fins diagnóstico e terapêutico.

As diferentes células tumorais compartilham propriedades únicas que as diferenciam das células normais das quais se originam, quer seja sob o ponto de vista genético ou fisiológico. A transformação oncogênica, em geral, corresponde a uma alteração no balanço entre a proliferação e a morte celular. Portanto, uma promissora alternativa terapêutica é a influência de anticorpos em mecanismos que interceptam a tumorigênise incluindo tanto a indução apoptótica, quanto a inibição da proliferação celular (BEN-KASUS et al., 2007).

A apoptose, ou morte celular programada, ocorre por meio de dois mecanismos: o extrínseco, ativado por citocinas (como TNF) e o intrínseco (ou mitocondrial), responsável pela liberação do citocromo e pela diminuição na atividade de proteínas da família Bcl-2. Ambos os mecanismos convergem para a ativação da cascata de proteases e caspases, as quais clivam moléculas regulatórias e estruturais. Diferentes anticorpos com aplicação terapêutica

encontram-se envolvidos nessa via de sinalização (BUBIEN et al., 1993). O *trastuzumab* é responsável por induzir a apoptose em tumores de mama com superexpressão de Her-2 e sua atividade pró-apoptótica tem sido atribuída à sua capacidade de inibir as vias de sinalização da MAP-quinase (proteínas quinases ativadoras de mitógenos) e Akt-quinase (proteína com atividade antiapoptótica em células com super-expressão de Her2) (CUELLO et al., 2001).

Portanto, a utilização de anticorpo e seus fragmentos demonstram a ascensão de uma trajetória promissora em ensaios clínicos. A combinação de estratégias e alvos nesse caso, além de um desafio, tem se mostrado como um futuro provável na luta contra o câncer.

Atualmente, o maior desafio no estudo da imunologia de tumores é a identificação de antígenos neoplásicos específicos ou associados capazes de desencadear uma resposta imune no paciente (PREUSS et al., 2002). Contudo, a imunogenicidade tumoral não se restringe a antígenos isolados, mas a múltiplos marcadores, envolvidos em inúmeros processos e cascatas de sinalização (SAHIN et al., 1995).

O rápido progresso das tecnologias genômicas e proteômicas tem permitido a busca por mudanças moleculares em doenças humanas, resultando em uma lista crescente de prováveis alvos avaliados como potenciais marcadores. Nesse sentido, as propriedades bioquímicas e fisiológicas dos anticorpos podem ser manipuladas, resultando em fragmentos de anticorpos capazes de detectarem uma série de estruturas (ROMER et al., 2011).

Portanto, outra aplicação para os fragmentos Fab é seu valor como diagnóstico. Durante os últimos anos, inúmeros fragmentos de anticorpos produzidos em bactérias têm se tornado alternativas potenciais para o imunodiagnóstico. De um modo geral, esses fragmentos podem se ligar a uma séria de antígenos como haptenos e proteínas, ampliando, assim, seu caráter clínico (AHMAD et al., 2012).

6 CITOQUERATINAS COMO ALVOS ANTIGÊNICOS NO CÂNCER DE MAMA

A diversidade de funções exercidas pelas células epiteliais é refletida pela expressão de diferentes pares de citoqueratinas (CK), as quais são responsáveis por proteger o tecido do estresse mecânico e de agir como plataformas de

sinalização. Essas moléculas emergem, portanto, como estruturas dinâmicas envolvidas em diversos processos como controle traducional, proliferação, transporte de organelas e transformação maligna; todos veiculados a padrões de fosforilação e associações moleculares (MAGIN et al., 2007).

Ensaios de imunohistoquímica são amplamente utilizados na determinação de parâmetros histopatológicos para o diagnóstico e classificação de tumores. Análises da expressão de receptores hormonais (como estrógeno e progesterona) assim como a detecção da super-expressão de Her2 são procedimentos de rotina em amostras de tumores invasivos da mama. Contudo, a marcação de citoqueratinas tem se tornado um fator adicional na categorização e conduta clínica desses tumores (MORIYA et al., 2006)

Esses filamentos intermediários do citoesqueleto são classificados como tipo I (CK9-CK20) e tipo II (CK1-CK8). As células epiteliais expressam pelo menos uma proteína de cada tipo, as quais ocorrem como heteropolímeros não-covalentes (TAO et al., 2008). Na glândula mamária, a expressão de citoqueratinas é fortemente regulada e se correlaciona à origem das células ductais, uma vez que morfologicamente as células são separadas em duas populações: as células glandulares luminais e as células basais mioepiteliais (BANKFALVI et al., 2004; CIOCCA et al., 2006).

No epitélio bilaminar da mama, CK8 e CK18 caracterizam as células diferenciadas, ao passo que CK5 e CK14 encontram-se expressas na porção basal proliferativa (BUHLER e SCHALLER, 2005). A expressão de CK7, CK17 e CK19 é variável, mas geralmente baixa (BECKER et al., 2002). Todas as citoqueratinas compartilham o mesmo domínio estrutural e, por mais de 20 anos, têm sido utilizadas como marcadores epiteliais no diagnóstico histopatológico do CM, em que cânceres basais expressam CK5, 6 e 14 e /ou 17 (fenótipo basal/mioepitelial) e tumores epiteliais expressam CK8,18 e 19 (MALZAHN et al., 1998). Uma observação chave presente em diferentes estudos é que a expressão dessas moléculas muda rapidamente durante os processos de diferenciação, injúria e metástase (MAGIN et al., 2007).

De fato, diferentes citoqueratinas são codificadas não somente de maneira específica do ponto de vista tecidual, como também apresentam um padrão de expressão celular diferenciado sugerindo sua participação em inúmeras funções

epiteliais. Nesse sentido, a estrutura secundária dessas proteínas é conservada. Entretanto, domínios presentes na região de cauda e cabeça dessas moléculas não possuem uma sequencia conservada de aminoácidos, apresentando apenas um padrão de similaridade de 57% e 33%, respectivamente, no caso, por exemplo, da citoqueratina 10 (CHEN et al., 2006)

A CK10 é uma citoqueratina do tipo I, que se encontra tipicamente expressa na membrana suprabasal pós-mitótica de queratinócitos (CHEN et al., 2006), substituindo as citoqueratinas 5 e 14 características do epitélio basal ativo (SANTOS et al., 2002). Essa presença específica da citoqueratina 10 tem relacionado sua função ao controle da proliferação celular (REICHELT e MAGIN, 2002).

Nesse contexto, essa molécula tem sido associada à agressividade de algumas neoplasias podendo estar relacionada a fatores prognósticos em câncer hepatocelular (YANG et al., 2008). Além disso, tem sido descrita em outros tumores malignos, sendo uma das proteínas mais comumente encontradas em metástases linfáticas (ZONG et al., 2012).

A expressão de citoqueratina 10 também foi detectada na camada mais diferenciada de carcinoma de células cervicais escamosas podendo estar relacionada à diferenciação do comportamento tumoral (VAN BOMMEL et al., 1994).De fato, os filamentos intermediários, como a CK10, além de desempenharem suas funções enquanto componentes do citoesqueleto afetam as células epiteliais à susceptibilidade tumoral. Contudo, suas reais funções em tumores de mama ainda não foram elucidadas.

Apesar dos notáveis avanços no conhecimento molecular dos tumores de mama, adotar um único marcador ou parâmetro na sua definição pode não ser o ideal. Consequentemente, diversos marcadores precisam ser incorporados em um sistema que melhor compreenda o caráter clínico dessa doença, fornecendo métodos diagnósticos, preditivos e prognósticos mais aplicáveis. Portanto, aliar variáveis ambulatoriais com biomarcadores moleculares provenientes de diferentes tecnologias certamente culminará com um sistema robusto e eficaz para a melhor compreensão dessa diversificada doença que é o Câncer de Mama.

REFERÊNCIAS BIBLIOGRÁFICAS

ARAUJO, TG. **Construção de uma biblioteca de anticorpos recombinantes (Fab) anti-carcinoma mamário e obtenção de fragmentos ligantes de antígenos tumorais por *Phage Display.*** 2009. 136f. Dissertação (Mestrado em Genética – Instituto de Genética e Bioquímica, Universidade Federal de Uberlândia, Uberlândia, 2009.

AHMAD, Z. A. et al. scFv antibody: principles and clinical application. **Clin Dev Immunol**, v. 2012, p. 980250. 2012.

AMOS, K. D. et al. Triple-negative breast cancer: an update on neoadjuvant clinical trials. **Int J Breast Cancer**, v. 2012, p. 385978. 2012.

AUSTIN, P. Will dAbs challenge mAbs? **Nature**, v. 341, p. 484-485. 1989.

AZZAZY, H. M. e HIGHSMITH, W. E., JR. Phage display technology: clinical applications and recent innovations. **Clin Biochem**, v. 35, Sep, p. 425-45. 2002.

AZZAZY, H. M. e HIGHSMITH, W. E. J. R. Phage display technology: clinical applications and recent innovations. **Clinical Biochemistry**, v. 35, p. 425-45. 2002.

BANKFALVI, A. et al. Different proliferative activity of the glandular and myoepithelial lineages in benign proliferative and early malignant breast diseases. **Mod Pathol**, v. 17, Sep, p. 1051-61. 2004.

BARBAS, C. F. et al. **Phage Display. A laboratory manual.** . New York: Cold Spring Harbor Laboratory Press, v. 2001

BARBAS, C. F. et al. **Phage Display: A laboratory manual**. New York: Cold Spring Harbor Laboratory, v. 2001

BECKER, M. et al. Sensitive PCR method for the detection and real-time quantification of human cells in xenotransplantation systems. **Br J Cancer**, v. 87, Nov 18, p. 1328-35. 2002.

BEN-KASUS, T. et al. Cancer therapeutic antibodies come of age: targeting minimal residual disease. **Mol Oncol**, v. 1, Jun, p. 42-54. 2007.

BENHAR, I. Biotechnological applications of phage and cell display. **Biotechnol Adv**, v. 19, p. 1-33. 2001.

BIRNBAUM, D. et al. Basal and luminal breast cancers: basic or luminous? **International Journal of Oncology**, v. 25, p. 249-258. 2004.

BOYLE, P. e LEVIN, B. **World Cancer Report** 150 cours Albert Thomas, 69372 Lyon Cedex 08, France, p. 510. 2008

BRASILEIRO FILHO, G. **Bogliolo Patologia**. Rio de Janeiro: Editora Guanabara Koogan S.A., v. 2006

BRATKOVIC, T. Progress in phage display: evolution of the technique and its application. **Cell Mol Life Sci**, v. 67, Mar, p. 749-67. 2010.

BRENTON, J. D. et al. Molecular classification and molecular forecasting of breast cancer: ready for clinical application? **J Clin Oncol**, v. 23, Oct 10, p. 7350-60. 2005.

BUBIEN, J. K. et al. Transfection of the CD20 cell surface molecule into ectopic cell types generates a Ca2+ conductance found constitutively in B lymphocytes. **J Cell Biol**, v. 121, Jun, p. 1121-32. 1993.

BUHLER, H. e SCHALLER, G. Transfection of keratin 18 gene in human breast cancer cells causes induction of adhesion proteins and dramatic regression of malignancy in vitro and in vivo. **Mol Cancer Res**, v. 3, Jul, p. 365-71. 2005.

CALLAGY, G. et al. Molecular classification of breast cancers using tissue microarrays. **Diagnostic Molecular Pathology**, v. 12, p. 27-34. 2003.

CARTER, P. J. Potent antibody therapeutics by design. **Nat Rev Immunol**, v. 6, May, p. 343-57. 2006.

CHEN, J. et al. An unexpected role for keratin 10 end domains in susceptibility to skin cancer. **J Cell Sci**, v. 119, Dec 15, p. 5067-76. 2006.

CHOI, Y. L. et al. Triple-negative, basal-like, and quintuple-negative breast cancers: better prediction model for survival. **BMC Cancer**, v. 10, p. 507. 2010.

CIOCCA, V. et al. Cytokeratin profiles of male breast cancers. **Histopathology**, v. 49, Oct, p. 365-70. 2006.

CLEMENTI, N. et al. Phage Display-based Strategies for Cloning and Optimization of Monoclonal Antibodies Directed against Human Pathogens. **Int J Mol Sci**, v. 13, p. 8273-92. 2012.

CO, M. S. e QUEEN, C. Humanized antibodies for therapy. **Nature**, v. 351, Jun 6, p. 501-2. 1991.

CUELLO, M. et al. Down-regulation of the erbB-2 receptor by trastuzumab (herceptin) enhances tumor necrosis factor-related apoptosis-inducing ligand-mediated apoptosis in breast and ovarian cancer cell lines that overexpress erbB-2. **Cancer Res**, v. 61, Jun 15, p. 4892-900. 2001.

CURIGLIANO, G. et al. Immunology and breast cancer: therapeutic cancer vaccines. **Breast**, v. 16 Suppl 2, Dec, p. S20-6. 2007.

DANTAS-BARBOSA, C. et al. Antibody phage display libraries: contributions to oncology. **Int J Mol Sci**, v. 13, p. 5420-40. 2012.

DE BRUIN, R. et al. Selection of high-affinity phage antibodies from phage display libraries. **Nat Biotechnol**, v. 17, Apr, p. 397-9. 1999.

DE HAARD, H. J. et al. A large non-immunized human Fab fragment phage library that permits rapid isolation and kinetic analysis of high affinity antibodies. **J Biol Chem**, v. 274, Jun 25, p. 18218-30. 1999.

DE MARCO, A. Biotechnological applications of recombinant single-domain antibody fragments. **Microb Cell Fact**, v. 10, p. 44. 2011.

DENARDO, D. G. e COUSSENS, L. M. Inflammation and breast cancer. Balancing immune response: crosstalk between adaptive and innate immune cells during breast cancer progression. **Breast Cancer Res**, v. 9, p. 212. 2007.

DESANTIS, C. et al. Breast cancer statistics, 2011. **CA Cancer J Clin**, v. 61, Nov-Dec, p. 409-18. 2011.

DUMITRESCU, R. G. e COTARLA, I. Understanding breast cancer risk - where do we stand in 2005? **Journal of Cellular and Molecular Medicine** v. 9, p. 208-221. 2005.

FILPULA, D. Antibody engineering and modification technologies. **Biomol Eng**, v. 24, Jun, p. 201-15. 2007.

FOULKES, W. D. et al. Triple-negative breast cancer. **N Engl J Med**, v. 363, Nov 11, p. 1938-48. 2010.

GALVAO, E. R. et al. Breast cancer proteomics: a review for clinicians. **J Cancer Res Clin Oncol**, v. 137, Jun, p. 915-25. 2011.

GEYER, F. C. et al. The role of molecular analysis in breast cancer. **Pathology**, v. 41, 1, Jan, p. 77-88. 2009.

GRUVER, A. M. et al. Molecular pathology of breast cancer: the journey from traditional practice toward embracing the complexity of a molecular classification. **Arch Pathol Lab Med**, v. 135, May, p. 544-57. 2011.

GUSTERSON, B. A. et al. Basal cytokeratins and their relationship to the cellular origin and functional classification of breast cancer. **Breast Cancer Research and Treatment**, v. 7, p. 143-8 2005.

HOLLIGER, P. e HUDSON, P. J. Engineered antibody fragments and the rise of single domains. **Nat Biotechnol**, v. 23, Sep, p. 1126-36. 2005.

JEONG, K. J. et al. Recombinant antibodies: engineering and production in yeast and bacterial hosts. **Biotechnol J**, v. 6, Jan, p. 16-27. 2011.

KEHOE, J. W. e KAY, B. K. Filamentous phage display in the new millennium. **Chem Rev**, v. 105, Nov, p. 4056-72. 2005.

KIM, S. J. et al. Antibody engineering for the development of therapeutic antibodies. **Mol Cells**, v. 20, Aug 31, p. 17-29. 2005.

KIPRIYANOV, S. M. e LE GALL, F. Generation and production of engineered antibodies. **Mol Biotechnol**, v. 26, Jan, p. 39-60. 2004.

LEE, B. L. et al. Breast cancer in Brazil: present status and future goals. **Lancet Oncol**, v. 13, Mar, p. e95-e102. 2012.

LEONG, A. S. e ZHUANG, Z. The changing role of pathology in breast cancer diagnosis and treatment. **Pathobiology**, v. 78, p. 99-114. 2011.

LEONG, S. S. J. e CHEN, W. N. Preparing recombinant single chain antibodies. **Chemical Engineering Science**, v. 63, p. 1401-1414. 2008.

LONBERG, N. Human antibodies from transgenic animals. **Nat Biotechnol**, v. 23, Sep, p. 1117-25. 2005.

LOW, N. M. et al. Mimicking somatic hypermutation: affinity maturation of antibodies displayed on bacteriophage using a bacterial mutator strain. **J Mol Biol**, v. 260, Jul 19, p. 359-68. 1996.

MAGIN, T. M. et al. Structural and regulatory functions of keratins. **Exp Cell Res**, v. 313, Jun 10, p. 2021-32. 2007.

MALHOTRA, G. K. et al. Histological, molecular and functional subtypes of breast cancers. **Cancer Biol Ther**, v. 10, Nov, p. 955-60. 2010.

MALZAHN, K. et al. Biological and prognostic significance of stratified epithelial cytokeratins in infiltrating ductal breast carcinomas. **Virchows Arch**, v. 433, Aug, p. 119-29. 1998.

MAYROSE, I. et al. A graph-based algorithm for epitope mapping using combinatorial phage-display libraries. **Nucleic Acid Research**, v. 35, p. 69-78. 2007.

MCPHERSON, K. et al. ABC of breast deseases.Breast cancer-epidemiology, risk factors, and genetics. **British Medical Journal**, v. 321, p. 624-8. 2000.

MEISNER, A. L. et al. Breast disease: benign and malignant. **Med Clin North Am**, v. 92, Sep, p. 1115-41, x. 2008.

MORIYA, T. et al. New trends of immunohistochemistry for making differential diagnosis of breast lesions. **Med Mol Morphol**, v. 39, Mar, p. 8-13. 2006.

NELSON, A. L. Antibody fragments: hope and hype. **MAbs**, v. 2, Jan-Feb, p. 77-83. 2010.

O'BRIEN, P. M. e AITKEN, R. **Methods in Molecular Biology: Antibody Phage Display - Methods and Protocols**. New Jersey: Human Press, v. 178. 2003

PELLETIER, J. e SIDHU, S. Mapping protein-protein interactions with combinatorial biology methods. **Curr Opin Biotechnol**, v. 12, Aug, p. 340-7. 2001.

PEROU, C. M. et al. Molecular portraits of human breast tumours. **Nature**, v. 406, Aug 17, p. 747-52. 2000.

PETRACCI, E. et al. Risk Factor Modification and Projections of Absolute Breast Cancer Risk. **J Natl Cancer Inst**, p. 2011.

PIGNOCHINO, Y. et al. Targeting EGFR/HER2 pathways enhances the antiproliferative effect of gemcitabine in biliary tract and gallbladder carcinomas. **BMC Cancer**, v. 10, p. 631. 2010.

PREUSS, K. D. et al. Analysis of the B-cell repertoire against antigens expressed by human neoplasms. **Immunol Rev**, v. 188, Oct, p. 43-50. 2002.

RATKAJ, I. et al. Integrated gene networks in breast cancer development. **Funct Integr Genomics**, v. 10, Mar, p. 11-9. 2010.

REICHELT, J. e MAGIN, T. M. Hyperproliferation, induction of c-Myc and 14-3-3sigma, but no cell fragility in keratin-10-null mice. **J Cell Sci**, v. 115, Pt 13, Jul p. 2639-50. 2002.

REIS-FILHO, J. S. e PUSZTAI, L. Gene expression profiling in breast cancer: classification, prognostication, and prediction. **Lancet**, v. 378, Nov 19, p. 1812-23. 2011.

ROMER, T. et al. Engineering antibodies and proteins for molecular in vivo imaging. **Curr Opin Biotechnol**, v. 22, Dec, p. 882-7. 2011.

RUSSEL, M. et al. Introduction to phage biology and phage display. In: T. Clackson e H. B. Lowman (Ed.). **Phage Display**. Oxford: Oxford University v. 266, 2004. Introduction to phage biology and phage display, p. 1-26

SAHIN, U. et al. Human neoplasms elicit multiple specific immune responses in the autologous host. **Proc Natl Acad Sci U S A**, v. 92, Dec 5, p. 11810-3. 1995.

SALLES, M. et al. [Diagnosis and treatment of congenital bronchopulmonary malformations. A review of 32 cases]. **Arch Pediatr**, v. 12, Dec, p. 1703-8. 2005.

SANTOS, M. et al. The expression of keratin k10 in the basal layer of the epidermis inhibits cell proliferation and prevents skin tumorigenesis. **J Biol Chem**, v. 277, May 24, p. 19122-30. 2002.

SERGEEVA, A. et al. Display technologies: application for the discovery of drug and gene delivery agents. **Adv Drug Deliv Rev**, v. 58, Dec 30, p. 1622-54. 2006.

SMITH, G. P. Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface. **Science**, v. 228, p. 1315-137. 1985.

SMITH, G. P. e PETRENKO, V. A. Phage Display. **Chem Rev**, v. 97, Apr 1, p. 391-410. 1997.

SORLIE, T. et al. Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. **Proc Natl Acad Sci U S A**, v. 98, Sep 11, p. 10869-74. 2001.

SORLIE, T. et al. Repeated observation of breast tumor subtypes in independent gene expression data sets. **Proc Natl Acad Sci U S A**, v. 100, Jul 8, p. 8418-23. 2003.

TAO, G. Z. et al. Monitoring of epithelial cell caspase activation via detection of durable keratin fragment formation. **J Pathol**, v. 215, Jun, p. 164-74. 2008.

VAN BOMMEL, P. F. et al. Expression of cytokeratin 10, 13, and involucrin as prognostic factors in low stage squamous cell carcinoma of the uterine cervix. **Cancer**, v. 74, Oct 15, p. 2314-20. 1994.

WALHOUT, A. J. e VIDAL, M. Protein interaction maps for model organisms. **Nat Rev Mol Cell Biol**, v. 2, Jan, p. 55-62. 2001.

WARK, K. L. e HUDSON, P. J. Latest technologies for the enhancement of antibody affinity. **Adv Drug Deliv Rev**, v. 58, Aug 7, p. 657-70. 2006.

WEISSER, N. E. e HALL, J. C. Applications of single-chain variable fragment antibodies in therapeutics and diagnostics. **Biotechnol Adv**, v. 27, Jul-Aug, p. 502-20. 2009.

YANG, X. R. et al. Cytokeratin 10 and cytokeratin 19: predictive markers for poor prognosis in hepatocellular carcinoma patients after curative resection. **Clin Cancer Res**, v. 14, Jun 15, p. 3850-9. 2008.

ZHANG, Q. et al. Guided selection of an anti-gamma-seminoprotein human Fab for antibody directed enzyme prodrug therapy of prostate cancer. **Cancer Immunol Immunother**, v. 56, Apr, p. 477-89. 2007.

ZONG, J. et al. Proteomic research progress in lymphatic metastases of cancers. **Clin Transl Oncol**, v. 14, Jan, p. 21-30. 2012.

CAPÍTULO 2

Anticorpo Fab humano capaz de reconhecer um epítopo conformacional da Citoqueratina 10 melhora o diagnóstico e prognóstico do Câncer de Mama

[Capítulo escrito de acordo com as normas exigidas pela revista Journal of the National Cancer Institute]

Title: Human Fab antibody targeting a cytokeratin-10 conformational epitope improves breast cancer diagnosis and prognosis

Authorship:

Thaise G. Araújo[1], Carlos E. Paiva[2], Rafael M. Rocha[3], Yara C.P. Maia[1], Angela A.S. Sena[1], Carlos Ueira-Vieira[1], Ana Paula C. Santos[1], Juliana F. Almeida[1], Paulo R. De Faria[4], Donizete W. Santos[5], Luanda Calábria[5], Tânia M. Alcântara[6], Fernando A. Soares[3], Luiz R. Goulart[1,7]

Affiliation:

[1]Federal University of Uberlandia, Institute of Genetics and Biochemistry, Nanobiotechnology Laboratory, Campus Umuarama, Bloco 2E, Sala 248, 38400-902, Uberlandia, MG, Brazil.

[2]Division of Breast and Gynecological Tumors, Department of Clinical Oncology, Barretos Cancer Hospital, Barretos, SP, Brazil.

[3]AC Camargo Cancer Hospital, São Paulo, SP, Brazil.

[4]Federal University of Uberlandia, Biomedical Institue, Uberlandia, MG, Brazil.

[5]Obstetric Division, Internal medicine, University Hospital, Federal University of Uberlandia, Uberlandia, MG, Brazil.

[6]Pathology Division, Internal medicine, University Hospital, Federal University of Uberlandia, Uberlandia, MG, Brazil.

[7]University of California Davis, Dept. of Medical Microbiology and Immunology, Davis, CA, USA.

Corresponding Author:

Luiz Ricardo Goulart, Federal University of Uberlandia, Institute of Genetics and Biochemistry, Laboratory of Nanobiotechnology, Campus Umuarama, Bloco 2E, 38400-902, Uberlandia, MG, Brazil. Phone: + 55 34 3218 2478, Fax: + 55 34 3218 2203. lrgoulart@ufu.br.

RESUMO

Atualmente não existem biomarcadores capazes de predizer o resultado ou evitar tratamentos desnecessários no câncer de mama (BC), e os marcadores de diagnóstico apresentam um comportamento variável, o que sugere uma maior complexidade e heterogeneidade dos subtipos de BC existentes. Nós caracterizamos como alvo biológico um novo anticorpo Fab em tecidos BC e avaliamos sua relevância clínica para o diagnóstico, prognóstico e estadiamento da doença.

Uma biblioteca combinatória de anticorpos Fab foi construída utilizando uma mistura dos transcritos de 20 pacientes com carcinoma ductal invasivo. A seleção por *phage display* contra tecidos BC de todos os estadiamentos da mama identificou o anticorpo FabC-4, que foi intensamente investigado por imunohistoquímica (IHC) em um *tissue microarray* gerado de uma coorte com 232 pacientes BC. O ligante de FabC-4 foi determinado por espectrometria de massas.

O FabC-4 foi selecionado baseado na sua elevada reatividade em todos os estádios de BC e seu poder de diferenciar de doenças benignas e controles saudáveis, com sensibilidade e especificidade significativa (70% e 62%, respectivamente). A sua expressão mais elevada em tecidos foi associada com BCs agressivos; por exemplo, menor idade, ausência do receptor de progesterona, graus histológicos maiores e fenótipos não-luminais e também foi identificado um subgrupo com bom prognóstico nos BCs triplo-negativo. O alvo biológico do FabC-4, identificado por espectrometria de massa, é um epítopo conformacional da citoqueratina-10 (CK10).

Nosso anticorpo específico de um epítopo de CK10 é o primeiro grande biomarcador bi-funcional tecido específico para o diagnóstico e classificação histopatológica de BC, o qual também estar associado com agressividade de BCs. Além disso, o anticorpo identifica um subgrupo de BCs triplo negativos com bom prognóstico. Seu papel no BCs devem ser abordados em estudos futuros.

Palavras chave: câncer de mama, anticorpos recombinantes, citoqueratinas

ABSTRACT

Background

Currently there are no biomarkers capable of predicting the outcome or avoiding unnecessary treatment in breast cancer (BC), and diagnostic markers have variable behavior, suggesting a higher complexity and heterogeneity of existing BC subtypes. We have characterized the biological target of a new Fab antibody in BC tissues and assessed its clinical relevance in diagnostics, disease staging and prognosis.

Methods

A Fab antibody combinatorial library was constructed by mixing transcripts from twenty patients with invasive ductal carcinoma. Phage Display selections against BC tissues from all stages led to the breast specific FabC-4 antibody, which was thoroughly investigated by immunohistochemistry (IHC) in a tissue microarray generated by a cohort of 232 BC patients. The FabC-4 ligand was determined by mass spectrometry.

Results

The FabC-4 was selected based on its high reactivity to all BC stages and discrimination power from benign diseases and healthy controls, with significant sensitivity and specificity (70% and 62% respectively). Its higher tissue expression was associated with aggressive BCs; i.e., younger age, lack of progesterone receptor, higher histological grades and non-luminal phenotypes, and it also identified a subset of good prognostic triple-negative BCs. Its biological target, identified through mass spectrometry, is a conformational epitope of Cytokeratin-10 (CK10).

Conclusion

A CK10-epitope specific antibody is the first bi-functional highly specific tissue biomarker for BC diagnosis and histopathological classification, which was also shown to be associated with aggressive BCs. In addition, the antibody identified a subset of triple negative BCs with good prognosis. Its role in BCs should be addressed in future studies.

INTRODUCTION

Breast cancer (BC) is the most frequent malignant tumor of women in North America [1], is the second leading cause of death, after lung cancer [2] and the parameters currently available are not sufficient to capture its individual complexity [3]. During the past decade, various genomics-based techniques have been applied with increasing success to the molecular characterization of breast tumors [4]. However, a heterogeneous disease, encompassing a wide variety of pathological entities in which 40% of the patients still succumb, highlights the need for new therapeutics strategies and identification of new targets [3, 5].

It is known that malignant transformation of cells often causes dramatic changes in the expression of cell surface molecules [6] and antibodies have proven to be an excellent paradigm for the design of high-affinity, protein-based binding reagents [7]. Monoclonal antibodies (mAb) are generated by either hybridoma technology or from combinatorial antibody libraries [6]. The construction and selection of antibody combinatorial libraries on filamentous phage surface became an alternative in search for antigen-specific clones without cross-reactivity, unequal expression of genes repertoire and yielding new applications in diagnosis and therapy [8-10]. A crucial advantage of this technology is the direct link between the experimental phenotype and its encapsulated genotype, which allows the evolution of the selected binders into optimized molecules [11, 12]. Several human combinatorial antibody libraries displayed on filamentous phage surface have been built to select antibodies against different antigens [13-16] including melanoma [17], colorectal [18] and prostate [6, 19, 20] cancer proteins.

Several lines of evidence indicate that stromal cell responses may promote progression to cancer and metastasis. To identify these probable factors involved in development of BC presenting in stromal cells, we analyzed a constructed BC-specific Fab library diversity and selected a breast-specific antibody clone, FabC-4, which recognizes a cytokeratin 10 (CK10) conformational epitope, and evaluated its applicability in a cohort of BC patients with long-term follow-up in order to associate its expression with clinical-pathological characteristics and survival.

METHODS

Study design and initial sample collection

This Project was carried out from 2008 to 2009 at the Nanobiotechnology Laboratory of the Federal University of Uberlandia (UFU) together with the Obstetric Service of University Hospital. The study protocol was approved by the Institutional Research Ethics Board (N. 176/2008), and an informed consent was obtained from all participants. All peripheral blood leukocytes (PBL) and tissues samples were obtained from patients that live in Uberlandia – MG (Brazil). The ethnic background was not recorded since the Brazilian population is highly heterogeneous and mixed. Peripheral blood samples were collected before surgery in a vaccutainer^{TM} tube containing K_2EDTA 7.2 mg, and maintained at 4ºC.

To construct an Fab combinatorial library we have obtained PBL from 20 women patients (mean age of 54 years) with ductal invasive BC grade I (5%), grade II (90%) and grade III (5%), submitted to mastectomy with no preoperative chemotherapy, radiation or hormonal therapy. Breast tissues from three patients diagnosed with ductal invasive BC (two classified as grade II and one as grade III, mean age of 52 years, mastectomized, and presenting more than 80% of malignant tissue) were used to perform selection of the phage display antibody library. Normal tissues from patients submitted to breast reduction surgery (mean age, 50 years), and with no familial history of breast cancer, were collected under an informed consent and were classified as a control group.

Construction of human Fab combinatorial library and selection by Phage Display

Total RNA was extracted from PBL of each patient by Guanidine Isothiocyanate extraction method [21] with minor modifications. RNAs were pooled in equimolar and four micrograms were mixed with 10 pmol of specific primers for amplifications of the heavy and light chain immunoglobulins [22] that were submitted to 70ºC for 10 minutes. The reverse transcription was performed with SuperScriptII Reverse Transcriptase (Invitrogen) according to manufacturer's instructions. The reaction was then terminated by heating to 70ºC for 15 min. First-strand cDNA derived from PBL of BC patients was used to generate the Fab

genes repertoire by PCR reactions and selection of Fab fragments was performed as described elsewhere [23]. Selection was performed by three cycles of selection against breast tumor tissues, after subtracting from normal tissue to avoid cross reactivity of clones. Soluble Fab antibodies were produced after selection, by transforming each individual selected clone into a TOP10 *E. coli* non-suppressor strain, as described elsewhere [23].

ELISA screening

In order to investigate BC antigen recognition, Immuno 96 Micro-Well™ (Nunc, Denmark) plates were coated with 1,0 µg/well of total poll protein extracted from normal, benign and tumor breast tissues in 100 µL of sodium bicarbonate buffer pH 7.4 ($NaHCO_3$), overnight at 4ºC. The plates were washed 3 times with PBST 0.05% and blocked with 5% slim milk-PBS for 3 h at room temperature. After washing, 100 µL of each culture supernatant were added to appropriate wells and incubated at room temperature for 2 hours. The plates were washed 5 times with PBST. HRP-conjugated rat anti-HA antibody was added to each well (100 µL, 1:1000 dilution) and the plates were incubated 1h at room temperature. The plates were washed 5 times with PBST and revealed with 100 µL of o-phenylenediaminen substrate (Sigma Aldrich). Reaction was stopped with 4N of sulfuric acid and the absorbance was read at 450nm.

Immunohistochemistry

After Fab selection, the affinity of mammary tissue epitopes was verified by immunohistochemical localization. Additional samples of breast adenocarcinoma, breast fibroadenoma and normal breasts from mammoplasty were processed and submitted to immunohistochemistry analyses, which were carried out by the following steps: sections were incubated with citrate buffer 6M for 1h at 90°C for antigen retrieval. The peroxidase blockage was performed with H_2O_2 3% in water for 30 minutes followed by blockage of unspecified sites with PBS/BSA 10% for 1h at room temperature. Then, the Fab addition (1:25) in tissue sections was sequentially performed overnight at 4°C. Control sections were incubated only with PBS. Immunoaffinity was analyzed by a mouse anti-HA conjugated to horseradish peroxidase (Sigma, 1:200 in PBS) for 1h at room temperature. Slides were then

revealed with diaminobenzidine substrate solution, counterstained with hematoxylin and observed in a light microscope (Olympus BX40). The photomicrographs were made by the *software* HLImage (Western Vision Software, USA). The final scores were obtained according to
immunostaining intensity in epithelial cells and were designated as negative (score 0 and 1) or positive (score 2 and 3). The analysis was carried out by four observers (RMR, TMA, PRF and FAS) and the samples were scored blinded with respect to clinical patient data. In case of discrepant recording, a consensus score was used.

Immunoprecipitation and protein sequencing

We performed immunoprecipitation of FabC-4 using Mouse Anti-His mAb Mag Beads (GenScript) according to manufacturer's instructions. Binding proteins were precipitated out of solution using the ProteoExtract kit (Calbiochem) and the protein pellet was left to dry overnight in a sterile fumehood. The lyophilized pellet was then resuspended in 50mM Ammonium bicarbonate (pH 8.0) and subjected to an in-solution tryptic digestion (Mike Myers, Cold Spring Harbor modified by Brett S. Phinney, UC Davis Proteomics Core). Digested peptides were then de-salted using aspire tips (Thermo-Fisher Scientific, RP30 tips) before being resuspended in loading buffer.

Digested peptides were analyzed using a LTQ-FT (Thermo Fisher Scientific) coupled with a MG4 paradigm HPLC (Michrom, Auburn, CA). The samples were loaded onto a Michrom cap trap (0.5x2mm) to be de-salted. The peptides were then separated using a Michrom Magic C18AQ (200 µm×150 mm) reversed-phase column and eluted using a gradient during a period of 60 minutes. Collision induced dissociation was applied to the peptide samples and data was acquired with an isolation width of 1, a normalized collision energy of 35 and a resolution of 50,000. The spray voltage on the Michrom captive spray was set to 1.8kV with a heated transfer capillary temperature of 200°C.
Raw data was analyzed using XTandem and visualized using Scaffold (Proteome Software, version 3.01). Samples were searched against Uniprot human (130,611 sequences) database appended with the cRAP (commonly found laboratory contaminants) and the reverse decoy databases.

Breast cancer sample characteristics

To validate the FabC-4, a population of BC patients' samples (n=232, mean age of 54 years range from 25 to 86) with long-term follow-up were obtained from A.C. Camargo Cancer Hospital (São Paulo, Brazil) and evaluated by immunohistochemistry in a previously constructed tissue microarray [24]. Patients were followed prospectively with a mean follow-up of 88.5 ± 63.1 months (3–227 months). All samples were from untreated patients before surgery. Patient's characteristics are described in Table 1.

Table 1. Patient characteristics (n=232).

Variable	Patients	
	No.	%
Menopausal status		
Premenopausal	98	42
Postmenopausal	134	58
Lymph node status		
cN0	74	32
cN1-3	158	68
Tumor stage		
cT1	19	8
cT2	101	43
cT3	48	21
cT4	64	28
Histological grading		
G1	44	19
G2	127	55
G3	61	26
ER status		
Negative	84	36
Positive	134	58
NA	14	6
PgR status		
Negative	156	67
Positive	65	28
NA	11	5
HER-2[a]		
Negative	166	72
Positive	22	9
NA	44	19
Molecular phenotype[b]		
Luminal	148	64
HER-2-enriched	16	7
Triple negative	48	21
NA	20	8
Chemotherapy		
No	39	17
Yes	193	83
Radiation therapy		
No	39	17
Yes	193	83
Hormone therapy		
No	126	54
Yes	106	46

Abbreviations: NA=not available; ER, estrogen receptor; HER2, human epidermal growth factor receptor 2; PgR, progesterone receptor. [a] HER2 status was considered as positive (score 3+) and negative (score 0–1+); scores 2+ were excluded from the analyses. [b] Cases were classified as luminal (ER+ and/or PgR+ with HER-2-), HER2-enriched (ER-/PgR-/HER2+), and triple negative (ER-/PgR-/HER2-).

Statistical Analysis

The chi-square test was applied to determine the strength of association between the categorical variables. Disease-free survival (DFS) and overall survival (OS) probabilities were calculated using the Kaplan–Meier method. The end-point for OS analysis was restricted to death due to breast cancer and for DFS analysis, the end-point was distant metastasis diagnosis. Kaplan–Meier survival curves for FabC-4 were also calculated in BC patients stratified according to molecular profile status. Multivariate analysis was carried out using Cox proportional hazards model. The following variables were included in the multivariate model according to their biological context relating to BC: age, ER, PgR, HER-2, histological grade, cT, cN, chemotherapy, hormonal therapy, radiation treatment as well as the FabC-4 status. The statistical analyses were carried out using SPSS version 15.0 (SPSS; Chicago, IL) for Windows. Statistical significance was considered when $P<0.05$.

RESULTS

Characterization of selected clones

The specificity of the selected soluble Fab's against breast cancer antigens was determined by ELISA assays. Eight clones demonstrated differential reactivity to the pool of proteins extracted from normal, benign tumor and breast cancer tissues (Figure 1A). All of them discriminated, by ANOVA test, benign from breast cancer tissues. Only D12 clone could not differentiated normal from BC samples. Nevertheless, only the FabC-4 clone was selected for further analyses, based on its reactivity ratios between cancer/benign and cancer/normal (Figure 1B), which were higher than the other clones. No positive signal was observed in the negative control (pComb3X without insert).

FabC-4 target identification and tissue microarray analysis

The antigen corresponding to the FabC-4 antibody was characterized as Cytokeratin 10 (CK10) by immunoprecipitation experiments. Captured sequences presented the highest number of sequence homologies in the mass spectrometry analysis. Additional western blot experiments in denatured conditions demonstrated that the FabC-4-binding epitope sequence is conformational, since

no staining was observed (data not shown). To confirm the target identification, a recombinant CK10 (Abnova) was obtained and submitted to ELISA with FabC-4, which showed a strong positive reactivity, similar to the reaction observed for BC tissue proteins and significantly different (P<0.01) from expression levels found in the other two groups (Figure 1C).

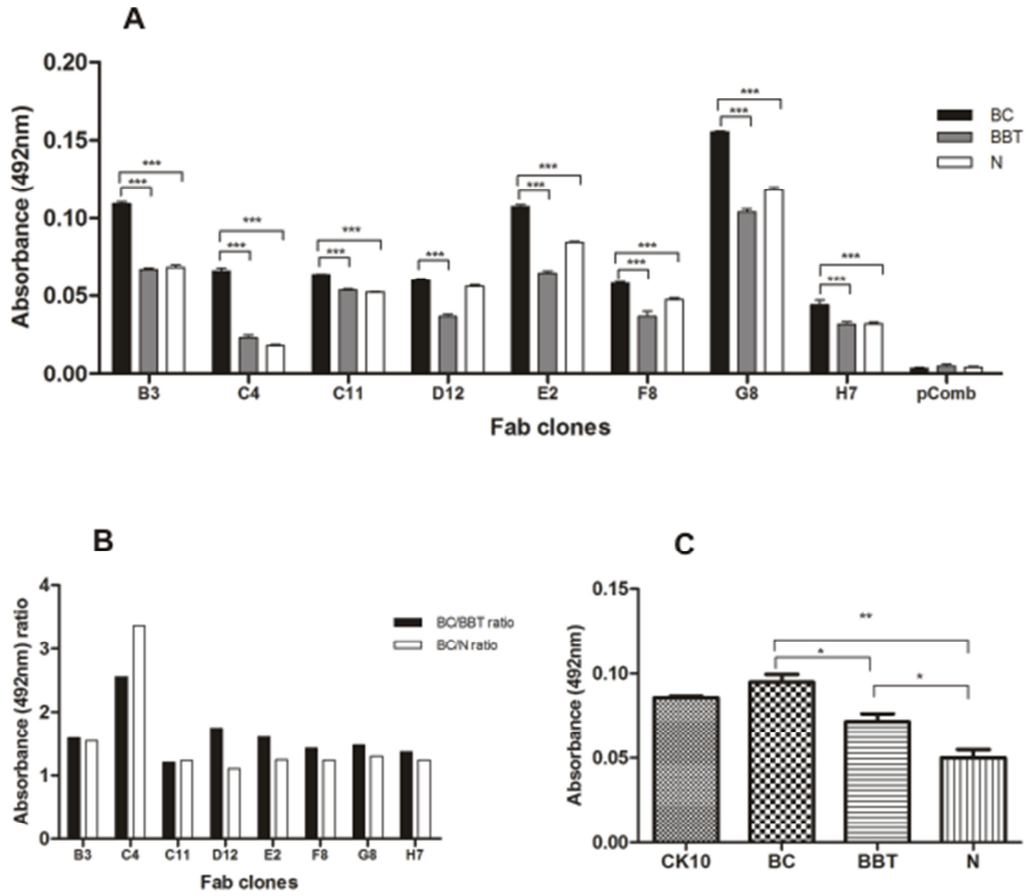

Figure 1: Evaluation of the binding selectivity for the induced clones using a pre-screening ELISA in total protein extracted from normal, benign and tumor tissue samples. Absorbance in 492nm is described in panel A with 8 reactive clones. ANOVA test demonstrated that all clones discriminated breast cancer from benign samples. FabC-4 clone was selected for additional procedures, based on its reactivity ratio between cancer/benign and cancer/control (B), which was higher than the other clones. ELISA assay between the recombinant CK10 and the FabC-4 antibody for antigen validation (C). Absorbance was significantly different between the three groups of proteins extracted from BC; BBT and N patients. BC protein did not differed from CK10 absorbance and was positive for FabC-4 detection. The other groups presented significantly lower absorbance compared to CK10 recombinant protein. BC: breast cancer; BBT: benign breast tumor; N: normal tissue. *p<0.05; **p<0.01; ***p<0.001.

Immunohistochemistry data of the FabC-4 is also displayed in Fig. 2. Immunostaining was significantly higher ($p=0.0002$) in BC compared to benign tumor, and increased expression levels were correlated with the invasive breast cancer tumors in comparison to breast benign tumor and normal tissue. Strong labeling was verified in the ducts of invasive carcinoma (Figure 2A), while the benign section showed a moderate immunoreactivity (Figure 2B) and none labeling was observed in normal breast tissue (Figure 2C) and reaction control sections (Figure 2D). Moreover, it was tested the cross-reaction with other cancer types such as prostate, stomach, pancreas and lymphoma, which presented none or a weak FabC-4 immunoreactivity (Figure 2E-H).

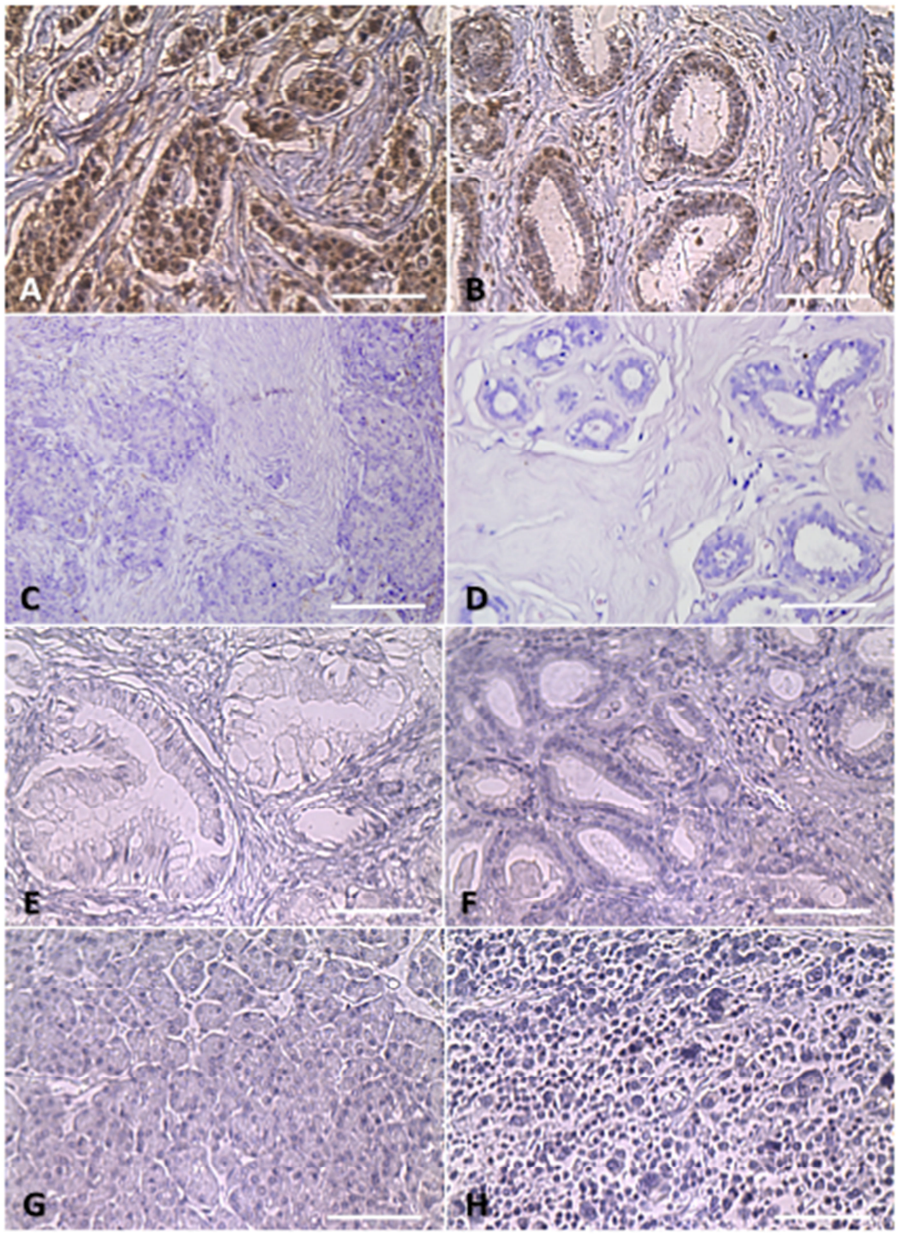

Figure 2: Immunoaffinity of FAbC-4 against breast cancer tissue antigens. (A) Invasive adenocarcinoma showing striking labeling in the nucleus and cytoplasm from ductal cells. (B) Moderate cytoplasmic immunoreactivity of ductal epithelial cells in benign breast tissue with fibroadenoma. (C) Mamoplasty sample tissue showing none labeling with FabC-4. (D) Negative control of immunohistochemistry assay. (E-H) None immunoreactivity was observed in other cancer types, such as prostate, stomach, pancreas and lymphoma. Counterstaining: Hematoxylin.

FabC-4 immunoreactivity in breast cancer and clinical-histopathological variables

The selected FabC-4 presented an overall accuracy of 61%, but its expression was increased during cancer development and reached a positivity of 88.5% in advanced BC stages.

Negative association between age at diagnosis and FabC-4 immunoreactivity rates was observed ($p=0.04$), since 84.2% of BC patients <40 years had FabC-4 expression in comparison with 63.6% of positivity from BC patients >60 years old (Table 2). Patients with absence of expression of ER and PgR had higher percentage of FabC-4 positivity, although only PgR analysis reached statistical significance ($p=0.09$ and $p=0.01$, respectively, Table 2). Histological grades were clearly associated with FabC-4, since its positivity rates were 59.1%, 63.8%, and 88.5% in GI, GII, and GIII BCs ($p<0.001$, Table 2). Regarding the molecular profile classification, luminal BCs presented lower number of FabC-4 immunoreactivity in comparison with non-luminal tumors. On the other hand, we could not observe any difference between Her2-enriched and TNBC (Table 2). Regarding menopausal status, HER-2 protein expression, and initial TNM stage, no statistical significant associations were observed (Table 2).

Table 2. FabC-4 exprssion detected by immunohistochemistry in breast tumour samples and the clinical–histopathological variables (n=232).

Variable	N	FabC-4		*p-value*
		Negative N (%)	Positive N (%)	
Age (years)				**0.04**
<40	34	6 (17.6)	28 (84.2)	
40-60	110	33 (30.0)	77 (70.0)	
>60	88	32 (36.4)	56 (63.6)	
Menopausal status				0.77
Pre	98	29 (29.6)	69 (70.4)	
Post	134	42 (31.3)	92 (68.7)	
ER				0.09
Positive	134	46 (34.3)	88 (65.7)	
Negative	84	20 (23.8)	64 (76.2)	
PgR				**0.01**
Positive	93	37 (39.8)	56 (60.2)	
Negative	127	30 (23.6)	97 (76.4)	
HER-2				1.00
Positive	22	6 (27.3)	16 (72.7)	
Negative	166	49 (29.5)	117 (70.5)	
Histological grading				**<0.001**
GI	44	18 (40.9)	26 (59.1)	
GII	127	46 (36.2)	81 (63.8)	
GIII	61	7 (11.5)	54 (88.5)	
Molecular profile				0.05
ER-/PgR-/HER-2-	48	9 (18.8)	39 (81.3)	
ER-/PgR-/HER-2+	16	3 (18.8)	13 (81.3)	
Luminal	148	52 (35.1)	96 (64.9)	
Tumor size (cT)				0.24
T_1	19	6 (31.6)	13 (68.4)	
T_2	101	35 (34.7)	66 (65.3)	
T_3	48	9 (18.8)	39 (81.3)	
T_4	64	21 (32.8)	43 (67.2)	
Lymph node (cN)				0.26
N_0	74	19 (25.7)	55 (74.3)	
N_{1-3}	158	52 (32.9)	106 (67.1)	
Distant metastasis (cM)				1.00
M_0	212	65 (30.7)	147 (69.3)	
M_1	20	6 (30.0)	14 (70.0)	
Recurrence				0.30
Yes	118	33 (28.0)	85 (72.0)	
No	111	38 (34.2)	73 (65.8)	
Death				0.16
Yes	113	30 (26.5)	83 (73.5)	
No	117	41 (35.0)	76 (65.0)	
Breast Cancer	232	71 (30.6)	161 (69.4)	**0.0002**
Benign Breast Disease	34	21 (61.8)	13 (38.2)	
Ovary Cancer	3	3 (100.0)	0	
Lymphoma	3	3 (100.0)	0	
Pancreas	3	3 (100.0)	0	
Prostate	3	3 (100.0)	0	
Stomach	3	3 (100.0)	0	

HER-2, human epidermal growth factor receptor-2; ER, estrogen receptor; PgR, progesterone receptor.

FabC-4 immunoreactivity and breast cancer outcomes

No association was observed between FabC-4 immunoreactivity and DFS and OS analysis (data not shown). As expected, significant associations were detected between the clinical outcome and the established prognostic factors (nodal status, clinical stage, histological grade, ER and PgR status, and molecular profile).

Multivariate analysis demonstrated that tumor size (cT) and lymph node status (cN) were the only independent prognostic factors for DFS (HR: 1.80; 95% CI: 1.36-2.26; *P*<0.001 and HR: 2.20; 95% CI: 1.26-3.75; *P*=0.005, respectively). Regarding OS, in addition to cT (HR: 1.7; 95% CI: 0.27-2.16; *P*<0.001) and cN (HR: 1.9; 95% CI: 0.09-3.34; *P*=0.023), histological grade also presented independent prognostic impact (HR: 1.50; 95% CI: 0.03-2.18; *P* = 0.032).

BC patients were stratified according to molecular profiles (luminal vs. HER2-enriched vs. TNBC) and Kaplan–Meier survival curves were calculated. No prognostic impact was observed regarding the FabC-4 status in the groups of luminal and also in the HER2-enriched BCs (data not shown).

However, in the group with TNBCs, FabC-4 status could differentiate cases with distinct outcomes. Tumors with FabC-4 expression showed significantly increased DFS and OS (p=0.01 and p=0.02, respectively, Table 3 and Fig. 3). The median DFS of TNBCs was 13 months and 74 months in the groups with FabC-4 negative and positive, respectively. Furthermore, median OS was 21 months and 84 months, in the groups with FabC-4 negative and positive, respectively.

Table 3. Disease-free survival and overall survival for analyses of TNM stage I-III breast cancer patients with TNBC molecular phenotype (n = 42).

Variable	n	DFS		*P*	OS		*P**
		5 years (%)	10 years (%)		5 years (%)	10 years (%)	
Age (years)				0.652			0.691
<40	8	37.5	37.5		37.5	37.5	
40-60	24	52.9	36.1		57.4	41.8	
>60	10	30.0	30.0		30.0	30.0	
Menopausal status				0.636			0.667
Pre	24	40.0	31.1		44.1	35.3	
Post	18	50.0	42.9		50.0	42.9	
Histological grading				0872			0.950
G1	1	100	0.0		100	0.0	
G2	19	41.4	41.4		46.3	40.5	
G3	22	44.1	33.0		43.5	38.0	
Tumor size (cT)				**0.019**			0.054
T_1	4	25.0	25.0		25.0	25.0	
T_2	14	55.6	46.3		53.4	44.5	
T_3	15	53.3	53.3		60.0	52.5	
T_4	9	22.2	0.0		22.2	11.1	
Lymph node (cN)				0.320			0.333
N_0	12	54.7	41.0		53.5	53.5	
N_{1-3}	30	40.0	32.3		42.9	31.7	
FabC-4				**0.01**			**0.02**
Positive	34	51.8	40.3		51.2	43.8	
Negative	8	12.5	12.5		25.0	12.5	

Significant values are in bold.

*p-values obtained by log-rank test.

DFS, disease-free survival; OS, overall survival.

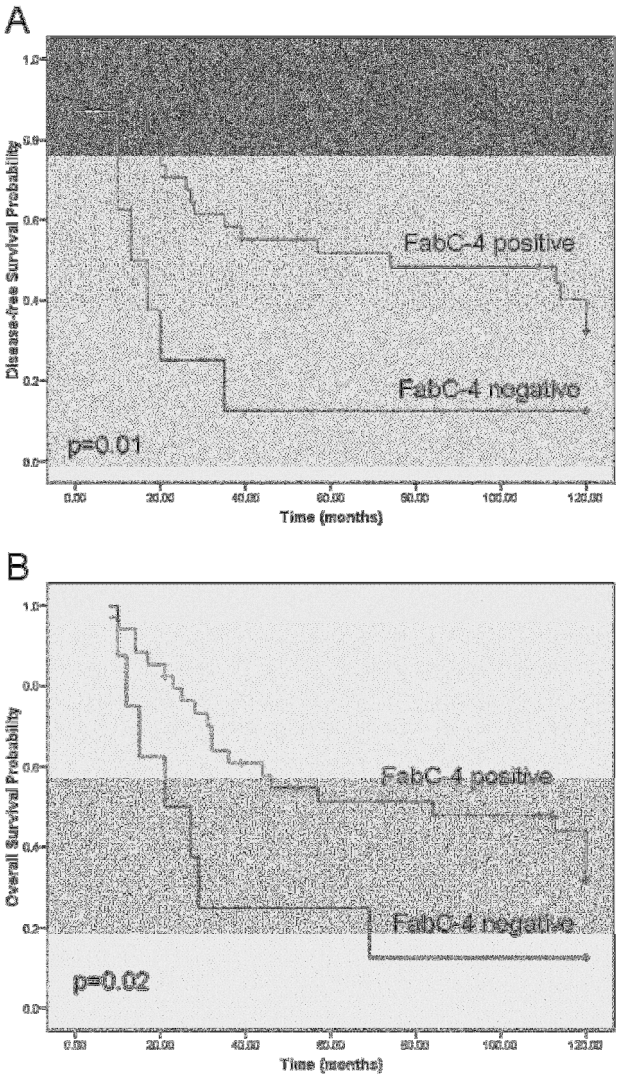

Figure 3: Disease-free survival and overall survival curves according to FabC-4 immunoreactivity. P values were determined by log-rank test. In (A) we present DFS and in (B) OS for Fab-C4 staining.

Multivariate analysis on the TNBC cases (n = 42) demonstrated that FabC-4 status is an independent prognostic factor for DFS and probably for OS. Lower risk of metastasis due to the disease was observed in FabC-4-positive tumor patients (HR = 0.33, 95%CI 0.13-0.87, p=0.026) and those submitted to radiotherapy (HR = 0.26, 95%CI 0.07- 0.94, p=0.04). Regarding OS, the only variable retained in the final model was FabC-4 (HR=0.40, 95%CI 0.15-1.03, p=0.05).

DISCUSSION

Currently, there are few biomarkers with prognostic significance in BC [3], but none can be used for diagnostic purposes. We have successfully selected by phage display a highly reactive antibody, FabC-4, with good sensitivity (70%) and specificity (62%) for diagnosis, good correlation with disease staging due to its increased expression during disease progression, and association with a subset of triple negative BCs with good prognosis. We have further characterized the specific ligand of the FabC-4 antibody, which was recognized as a conformational epitope of the CK10.

The FabC-4-ligand is breast specific, and its presence in some patients with benign diseases may be an indication of a pre-neoplastic disease without significant morphology alterations. On the other hand, the absence of the biomarker in tumor tissues may be due to the heterogeneity of the disease and some of the alterations cannot be explained by post-translational modifications of the CK10. However, this is the first potential biomarker for breast cancer diagnosis and histological classification. Interestingly, CK10 has been associated with proliferation control [25] and other cancers, and is one of the most common proteins in lymphatic metastases of cancers revealed by proteomic and protein functional studies [26]. CK10 has also been associated with poor prognosis in hepatocellular carcinoma regardless of tumor-node-metastasis stage, and vascular invasion [27]. Expression of cytokeratin 10 is also present in the more differentiated areas of squamous cell cervical carcinoma. However, these differentiation-related markers are not detectable in all squamous cell cervical carcinoma. This variable expression could be related to a difference in tumor behavior [28].

But because of the high specificity found in breast tissues in our study, we cannot rule out the possibility that conformational changes in CK10 may be associated with loss of function, as demonstrated in a CK10-null mouse model elsewhere [29], which has shown that CK10 is downregulated in squamous cell carcinomas and it is absent in proliferating cells *in vivo*, linking CK10 functions to both cellular architecture and cell cycle control.

Phage antibody library has been used before to generate high-affinity antibodies against previously defined tumor-associated antigens such as-CEA and

c-erb-2 [30-32], but were performed against specific ligands, different from our subtractive approach with unknown antigen target, which resulted in several tissue markers. The selected FabC-4 presented an overall accuracy of 61%, but its expression was increased during cancer development and reached a positivity of 88.5% in advanced BC stages.

Because our antibody showed a gradual immunoaffinity according to histopathological grade of mammary gland ducts with invasive carcinoma, and the CK10 epitope ligand showed significant protein expression in tumor tissues when compared to benign and normal tissues, it is expected that CK10 may also show differential expression during cancer progression with high tissue immunoreactivity to undifferentiated ducts, and weak or no reactivity to differentiated ducts from infiltrative adenocarcinoma or normal tissue from mammoplasty. The low expression in normal tissues may be due to the lack of post-translational modifications, which may play a critical role in the malignant transformation.

Challenging situations of metastatic cancers with unknown primary is very common, and deserves the utilization of breast-specific markers for differentiating BC from non-breast tissues. In this sense, ER, mammaglobin and gross cystic disease fluid protein-15 (GCDFP-15) are widely accepted biomarkers for immunohistochemistry [33]. Cases of metastatic TNBC are even more difficult for the pathologist, since those markers are less expressed [34].

ER, PR, HER-2, and Ki-67 protein expression are routinely evaluated in order to classify BCs into different molecular phenotypes, namely luminal A, luminal B, HER-2-enriched and TNBC [35]. Though widely used in clinical practice these biomarkers are not capable to capture the complexity of BC. TNBC represents a subset of aggressive tumors accounting for 15% to 20% of newly diagnosed BC cases [36]. Potential therapeutic targets are likely to be identified while the heterogeneity of TNBC is better defined [37]. In the present study, FabC-4 clone was associated with more aggressive tumors; i.e., those younger patients, with lack of PgR expression, higher histological grades and non-luminal BCs. Interestingly, in the subset of known aggressive TNBCs, FabC-4 was a good prognostic marker.

The major limitation of our study is regarding the sub-analysis of FabC-4 prognostic impact in TNBCs, since the number of patients evaluated was very

small. However, the hazard ratios were about 0.3 to 0.4 and the findings reached statistical significance even after multivariate analysis.

In conclusion, the CK10-epitope specific Fab antibody is the first bi-functional highly specific breast tissue biomarker, which can be used for BC diagnosis and prognosis, and it was also associated with a subset of triple negative BCs with good prognosis. Its role in BCs should be addressed in future studies.

Acknowledgements

The authors would like to thank the financial support by CNPq, CAPES, Ministry of Health (Department of Science and Technology, DECIT) and FAPEMIG. We would like to thank the Histopathology Laboratory of the Federal University of Uberlandia for providing the immunhistochemistry assays. We are also grateful to Prof. Dr. Andréia Q. Maranhão (University of Brasilia, Brazil) for providing the pComb3X vector.

Financial Support:

This study was supported by grants from National *Council* for the Improvement of Higher Education - CAPES, Brazil (T.G.A. scholarship), National *Council* for Scientific and Technological Development – CNPq (577579/2008-8) and FAPEMIG, Brazil (APQ-02613-10) (Dr Goulart).

REFERENCES

1. DeSantis C, Siegel R, Bandi P*, et al.* Breast cancer statistics, 2011. CA Cancer J Clin 2011;61(6):409-18.
2. Parkin DM, Bray F, Ferlay J*, et al.* Global cancer statistics, 2002. CA Cancer J Clin 2005;55(2):74-108.
3. Geyer FC, Marchio C, Reis-Filho JS. The role of molecular analysis in breast cancer. Pathology 2009;41(1):77-88.
4. Latterich M, Abramovitz M, Leyland-Jones B. Proteomics: new technologies and clinical applications. Eur J Cancer 2008;44(18):2737-41.

5. Hanby AM. The pathology of breast cancer and the role of the histopathology laboratory. Clin Oncol (R Coll Radiol) 2005;17(4):234-9.
6. Popkov M, Rader C, Barbas CF, 3rd. Isolation of human prostate cancer cell reactive antibodies using phage display technology. J Immunol Methods 2004;291(1-2):137-51.
7. Holliger P, Hudson PJ. Engineered antibody fragments and the rise of single domains. Nat Biotechnol 2005;23(9):1126-36.
8. Kim SJ, Park Y, Hong HJ. Antibody engineering for the development of therapeutic antibodies. Mol Cells 2005;20(1):17-29.
9. Ben-Kasus T, Schechter B, Sela M*, et al.* Cancer therapeutic antibodies come of age: targeting minimal residual disease. Mol Oncol 2007;1(1):42-54.
10. Hall PS, Cameron DA. Current perspective - trastuzumab. Eur J Cancer 2009;45(1):12-8.
11. Azzazy HM, Highsmith WE, Jr. Phage display technology: clinical applications and recent innovations. Clin Biochem 2002;35(6):425-45.
12. Smith GP, Petrenko VA. Phage Display. Chem Rev 1997;97(2):391-410.
13. Griffiths AD, Williams SC, Hartley O*, et al.* Isolation of high affinity human antibodies directly from large synthetic repertoires. EMBO J 1994;13(14):3245-60.
14. Pansri P, Jaruseranee N, Rangnoi K*, et al.* A compact phage display human scFv library for selection of antibodies to a wide variety of antigens. BMC Biotechnol 2009;9:6.
15. de Haard HJ, van Neer N, Reurs A*, et al.* A large non-immunized human Fab fragment phage library that permits rapid isolation and kinetic analysis of high affinity antibodies. J Biol Chem 1999;274(26):18218-30.
16. Wu BP, Xiao B, Wan TM*, et al.* Construction and selection of the natural immune Fab antibody phage display library from patients with colorectal cancer. World J Gastroenterol 2001;7(6):811-5.
17. Cai X, Garen A. Anti-melanoma antibodies from melanoma patients immunized with genetically modified autologous tumor cells: selection of specific antibodies from single-chain Fv fusion phage libraries. Proc Natl Acad Sci U S A 1995;92(14):6537-41.

18. Somers VA, Brandwijk RJ, Joosten B*, et al.* A panel of candidate tumor antigens in colorectal cancer revealed by the serological selection of a phage displayed cDNA expression library. J Immunol 2002;169(5):2772-80.
19. Mintz PJ, Kim J, Do KA*, et al.* Fingerprinting the circulating repertoire of antibodies from cancer patients. Nat Biotechnol 2003;21(1):57-63.
20. Zhang Q, Zhang SH, Su MQ*, et al.* Guided selection of an anti-gamma-seminoprotein human Fab for antibody directed enzyme prodrug therapy of prostate cancer. Cancer Immunol Immunother 2007;56(4):477-89.
21. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 1987;162(1):156-9.
22. Marks JD, Hoogenboom HR, Bonnert TP*, et al.* By-passing immunization. Human antibodies from V-gene libraries displayed on phage. J Mol Biol 1991;222(3):581-97.
23. Andris-Widhopf J, Steinberger P, Fuller R*, et al.* Generation of human Fab antibody libraries: PCR amplification and assembly of light- and heavy-chain coding sequences. Cold Spring Harb Protoc 2011;2011(9).
24. Nagai MA, Fregnani JH, Netto MM*, et al.* Down-regulation of PHLDA1 gene expression is associated with breast cancer progression. Breast Cancer Res Treat 2007;106(1):49-56.
25. Reichelt J, Magin TM. Hyperproliferation, induction of c-Myc and 14-3-3sigma, but no cell fragility in keratin-10-null mice. J Cell Sci 2002;115(Pt 13):2639-50.
26. Zong J, Guo C, Liu S*, et al.* Proteomic research progress in lymphatic metastases of cancers. Clin Transl Oncol 2012;14(1):21-30.
27. Yang XR, Xu Y, Shi GM*, et al.* Cytokeratin 10 and cytokeratin 19: predictive markers for poor prognosis in hepatocellular carcinoma patients after curative resection. Clin Cancer Res 2008;14(12):3850-9.
28. van Bommel PF, Kenemans P, Helmerhorst TJ*, et al.* Expression of cytokeratin 10, 13, and involucrin as prognostic factors in low stage squamous cell carcinoma of the uterine cervix. Cancer 1994;74(8):2314-20.

29. Relchelt J, Magin TM. Hyperproliferation, induction of c-Myc and 14-3-3 sigma, but no cell fragility in keratin-10-null mice. Journal of Cell Science 2002;115(13):2639-2650.
30. Schier R, Bye J, Apell G*, et al.* Isolation of high-affinity monomeric human anti-c-erbB-2 single chain Fv using affinity-driven selection. J Mol Biol 1996;255(1):28-43.
31. Begent RH, Verhaar MJ, Chester KA*, et al.* Clinical evidence of efficient tumor targeting based on single-chain Fv antibody selected from a combinatorial library. Nat Med 1996;2(9):979-84.
32. Osbourn JK, Field A, Wilton J*, et al.* Generation of a panel of related human scFv antibodies with high affinities for human CEA. Immunotechnology 1996;2(3):181-96.
33. Bhargava R, Beriwal S, Dabbs DJ. Mammaglobin vs GCDFP-15: an immunohistologic validation survey for sensitivity and specificity. Am J Clin Pathol 2007;127(1):103-13.
34. Litzenburger BC, Creighton CJ, Tsimelzon A*, et al.* High IGF-IR activity in triple-negative breast cancer cell lines and tumorgrafts correlates with sensitivity to anti-IGF-IR therapy. Clin Cancer Res 2011;17(8):2314-27.
35. Goldhirsch A, Wood WC, Coates AS*, et al.* Strategies for subtypes--dealing with the diversity of breast cancer: highlights of the St. Gallen International Expert Consensus on the Primary Therapy of Early Breast Cancer 2011. Ann Oncol 2011;22(8):1736-47.
36. Bauer KR, Brown M, Cress RD*, et al.* Descriptive analysis of estrogen receptor (ER)-negative, progesterone receptor (PR)-negative, and HER2-negative invasive breast cancer, the so-called triple-negative phenotype: a population-based study from the California cancer Registry. Cancer 2007;109(9):1721-8.
37. Metzger-Filho O, Tutt A, de Azambuja E*, et al.* Dissecting the heterogeneity of triple-negative breast cancer. J Clin Oncol 2012;30(15):1879-87.

CAPÍTULO 3

Interação entre Citoqueratina 18 e Anexina A1 no Câncer de Mama: desequilíbrio na sinalização e arquitetura celular

[Capítulo escrito de acordo com as normas exigidas pela revista Molecular Biology Reports]

Title: Dynamic dialogue between Cytokeratin 18 and Annexin A1 in breast cancer: cell architecture and signaling disequilibrium.

Authorship

Thaise G Araújo[1], Karina Marangoni[1], Yara CP Maia[1], Galber R Araújo[1], Tânia M Alcântara[2], Patrícia T Alves[1], Carlos Ueira-Vieira[1], Luiz R Goulart[1,3*]

Affiliation

[1]Laboratory of Nanobiotechnology, Institute of Genetics and Biochemistry, Federal University of Uberlandia, Uberlandia, MG, Brazil.

[2]Departament of Pathology, Clinical Hospital of Uberlandia, Federal University of Uberlandia, Uberlandia, MG, Brazil.

[3]Department of Medical Microbiology and Immunology, University of California Davis, Davis, CA, USA.

RESUMO

A sinalização celular é mediada por proteínas associadas às citoqueratinas (CKs), como a anexina A1 (ANXA1), um ligante do complexo CK18/CK8. A ANXA1 tem um papel central nas respostas celulares e imunológicas e em conjunto com CK18, tem sido implicada em vários processos relacionados com a transformação maligna do câncer da mama (CM). Nosso objetivo foi demonstrar como essa interação pode estar ligada ao desenvolvimento do CM. Nós investigamos os níveis transcricionais, a expressão de proteínas e a sua distribuição para os dois alvos em tecidos da mama de 92 pacientes (42 CMs e 50 doenças benignas), através de qPCR e imunohistoquímica, respectivamente. Os mRNAs de ANXA1 e CK18 foram inversamente correlacionados, e a sua razão em cada estágio TNM foram significativamente diferentes em CM e doenças benignas (OR = 5,62). Estas diferenças não refletem os níveis de proteína do tecido, mas foi observada uma distribuição dicotômica em tecidos tumorais, de forma diferente da esperada co-localização observada durante a homeostase celular, indicando modificações pós-traducionais diferenciadas nas células de CM. O desequilíbrio dos níveis de transcrição entre ANXA1/CK18 e as alterações na distribuição no tecido são evidências da perda da sua interação e remodelamento tecidual, o que sugere

defeitos na arquitetura celular e interrupção da sinalização celular, eventos críticos na iniciação e progressão do CM. A quebra do diálogo entre ANXA1 e CK18 em tecidos normais da mama pode desempenhar um papel crítico no desenvolvimento do CM, e em conjunto podem ser usadas como alvos combinados para o diagnóstico do CM.
Palavras-chave: Anexina A1/ Citoqueratina 18/ Câncer / imunohistoquímica/ níveis de expressão de mRNA

ABSTRACT

Cytokeratins (CKs) constitute the cytoskeletal network and are regulated by post-translational modifications, acting not only as a mechanical support, but also in cell signaling and regulatory processes. Signaling is mediated by CK-associated proteins, like Annexin A1 (*ANXA1*), a ligand of the *CK18*/*CK8* complex. *ANXA1* has a pivotal role in cellular and immunological responses, and together with *CK18* have been implicated in several processes related to malignant transformation in breast cancer (BC). Our aim was to demonstrate how their interaction might be linked to BC development. We have investigated transcript levels, protein expression and distribution for both targets in breast tissues of 92 patients (42 BCs and 50 benign diseases) through qPCR and immunohistochemistry, respectively. *ANXA1* and *CK18* mRNAs were inversely correlated, and their ratio in each TNM stage has significantly differentiated BC from benign diseases (OR=5.62). These differences did not mirror tissue protein levels, but a significant dichotomous protein distribution in tumor tissues was observed, differently from the expected co-localization observed during cell homeostasis, indicating differential post-translational modifications in BC cells. The disequilibrium of transcriptional levels between *ANXA1*/*CK18* and alterations in their tissue distribution are evidences of loss of their interaction and tissue remodeling, which suggest cell architecture defects and disruption of cell signaling, critical events in BC initiation and progression. The broken dialogue between *ANXA1* and *CK18* in normal breast tissues may play a critical role in BC development, and together may be used as combined targets for BC diagnostics.
Keywords: Annexin A1/ Cytokeratin 18/ breast cancer/ immunohistochemistry/ mRNA expression levels

INTRODUCTION

The intermediate filaments (IF) are cytoskeletal structures that function not only as mechanical support, but also are dynamically involved in cell signaling pathways, and are actively regulated by phosphorylation and other posttranslational modifications, determining either cell survival or apoptosis[1]. The IF network in simple glandular epithelial cells predominantly consists of heterotypic complexes of cytokeratin 8 (*CK8*) and cytokeratin 18 (*CK18*). In cancer, their persistent expression is accompanied by changes in cell morphology and alterations in the IF network[2].

Among several important signaling molecules, we have focused on Annexin A1 (*ANXA1*) that has been shown to be an IF-associated protein, specifically linked to *CK8* and *CK18*[3]. *ANXA1* is a calcium- and phospholipid-binding protein involved in many membrane-related events, such as membrane organization domains and membrane-cytoskeleton signaling[4]. *ANXA1* also seems to be a key molecule involved in the development of many types of cancer, and a modulator of the epithelial-mesenchymal transition (EMT) associated with highly invasive breast carcinomas (BC)[5].

However, *ANXA1* is a part of a complex and unknown network, and besides its function as membrane domains organizer, it also provides recruitment platforms for proteins with which it interacts. Interestingly, cytoskeletal disruptive drugs have anti-inflammatory[6] and anti-proliferative roles[7-9], and similarly *ANXA1* also functions as an anti-inflammatory, anti-proliferative and apoptotic molecule[10,11], through post-translational modifications[12].

It has been shown that CKs expression is tightly regulated and correlates with the origin of the cells in in the ducts of mammary glands[13], and *CK8*/*CK18* are co-localized with *ANXA1* in the human alveolar squamous epithelial cell line A549[3]; however, it is not known their joint distribution in benign and breast cancer human tissues. CKs have been recognized for more than 20 years as epithelial markers in histopathological diagnostics, in which basal like cells express CK5, CK6, and CK14 and /or CK17 (basal/myoepithelial phenotype) and luminal-like express *CK8*, *CK18*, and CK19[14-16]. Two key observations of many studies is that CK expression changes rapidly during differentiation, tissue injury, and metastasis[17], and despite the identification of *ANXA1* as one of several cellular

proteins that are differentially expressed during the progression of tumors, its role in the carcinogenesis has not yet been elucidated[18].

Therefore, we hypothesize that both *CK18* and *ANXA1* may play a far more dynamic and joint role in the tumor development and progression than thought before, and we believe that the link between them may provide novel clues and approaches to the current BC characterization[4,19-21]. Considering that most BCs are tightly linked to the *CK18* profile in tissues, and that *ANXA1* may have a great relevance in the cell signaling process through its association with *CK18*, we characterized the transcript and protein levels of both molecules, as well as their distribution in benign and tumor tissues, and showed striking differences in their transcript ratios and protein distribution in tumor tissues. We present important evidences of concerted action of both molecules in benign and tumor tissues, and the loss of homeostasis between them may represent a link of important cellular and molecular events for tumor initiation and progression.

MATERIALS AND METHODS

Study design and sample collection

Total of 92 patients were selected from the Obstetric Service at University Hospital at Federal University of Uberlandia, and grouped into two patients groups: 42 BCs and 50 with benign breast diseases (BBD). The Ethics Committee of the Institutional Research Board approved all procedures, under the number 176/2008, and an informed consent was obtained from all participants.

The surgical procedures of untreated patients consisted of radical mastectomy or quadrantectomy, depending on the size of the tumor and breast anatomic characteristics.

The average age of the patients investigated was 47.7 years (range 31-89 years) for BC group and 46.8 years (range 18-80 years) for BBD group. There were 12 (29%), 18 (43%), 5 (12%), and 7 (16%) breast tumors classified as TNM stages T_1, T_2, T_3 and T_4, respectively. The histological grading according to the Nottingham system were grade I (GI) in 4 (10%), GII 24 (57%), and GIII in 14 (33%).

Hormone receptors, estrogen receptor (ER) was positive in 28 (67%), negative in 9 (21%) and not evaluated in 5 (12%); progesterone receptor (PgR) was positive in 27 (64%), negative in 10 (24%) and not analyzed in 5 (12%). HER2 status was considered as positive (score 3+) and negative (score 0–1+); scores 2+ were excluded from the analyses. HER-2 status were positive in 12 cases (29%), negative in 25 (60%) and score 2+ or not analyzed in other 5 (11%).

Quantitative RT-PCR and Immunohistochemistry

For transcriptional analysis, RNA was extracted using the *Trizol* reagent (Invitrogen) according to the manufacturer's recommendations and the *CK18, ANXA1,* and *B2M (B*-2-microglobulin) transcripts were analyzed by qPCR with SybrGreen detection in an ABI PRISM 7300 (Applied Biosystems). The primers' sequences were: GATTCAGATGCCAGGGCCT and CACTCTGCGAAGTTGTGGAT for ANXA1; GCTCTGGGTTGACCGTGG and GTGGTGCTCTCCTCAATCTGC for *CK18;* CCTGCCGTGTGAACCATGT and GCGGCATCTTCAAACCTCC for *B2M*.

For protein detection of *ANXA1* and *CK18* and their tissue distribution, immunohistochemistry were carried out in Tissue MicroArrays (TMA) by using monoclonal antibodies for *ANXA1* (BD; 1:250) and for *CK18* (Cell Marque; 1:50), and the procedures were performed according to standard protocols with EnVisioned+System HRP (Dako). The final scores were obtained according to immunostaining intensity in epithelial cells and were designated as negative (score 0) or positive (score 1–3). The analysis was carried out by two observers (TMA and LRG) and the samples were scored blinded with respect to clinical patient data. In case of discrepant recording, a consensus score was used.

Statistical analysis

For statistical analysis we used Mann-Whitney test, Kendall's rank correlation (τ), McNemar's test, and Spearman's correlation for clinical parameters; patient age at diagnosis, TNM system staging, Scarff-Bloom-Richardson (SBR) grading, and hormonal status. Statistical significance was considered when $P<0.05$. The statistical analyses were carried out using

GraphPad Prism 5 (GraphPad Software Inc., La Jolla, CA) and SPSS version 17.0 (SPSS; Chicago, IL).

RESULTS

ANXA1 and *CK18* gene expression levels are in disequilibrium in breast cancer

No difference was observed for *ANXA1* transcripts between breast cancer and benign tissues, but lower *CK18* mRNA levels were detected in BC (P = 0.02) (Figure 1A). Relative quantification of *CK18* mRNA levels was 2.08-fold higher in BBD than in BC. A detection limit was estimated for *CK18* and *ANXA1* gene according to maximal differences between BC and BBD groups in their medians and percentiles. These limits were used to calculate the odds ratios values. The cut-off value for the relative levels of transcript revealed a down regulation of CK expression in BC samples (Figure 1B).

However, the most striking differences were observed for *ANXA1*:*CK18* ratios in breast cancer stages when compared to benign tissues (P<0.05) (Figure 1A). Considering the significant ratios observed between each tumor stage and BBD (>1.5 or <0.5), which indicated disequilibrium between markers, we have obtained a significant odds ratio of 5.62-fold towards BC occurrence ($CI_{95\%}$ 2.1 – 15.1, P=0.0009).

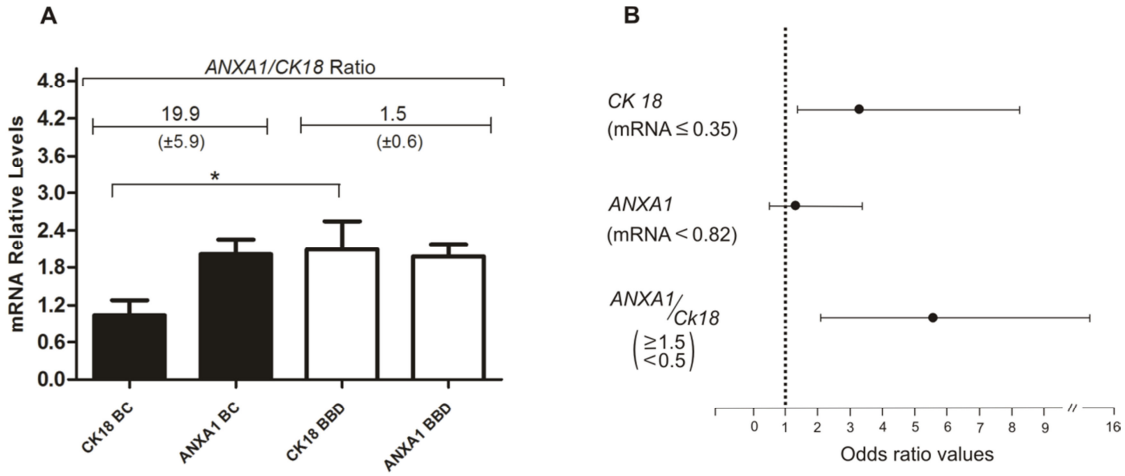

Figure 1: *ANXA1* and *CK18* mRNA expression levels, their ratios and odds ratios obtained in the comparison between BC and BBD. Relative quantification of *ANXA1* and *CK18* messenger RNA (mRNA) in breast tissues and benign samples and *ANXA1/CK18* transcripts ratio **(A)**. The values above the bars represent ANXA1/CK18 ratio for each histopathological classification and standard deviation (±)In **(B)** the Odds ratios estimated according to the detection limit for each marker. The values were calculated for Breast Cancer vs Benign Breast Disease

(*) P<0.05

Dichotomous *ANXA1* and *CK18* expression and distribution during cancer development and progression

A significant moderate negative correlation between CK18 and ANXA1 protein levels was found in breast samples (r = -0.31, P = 0.01). Interestingly, although *CK18* transcript levels are generally reduced across tumor stages, its protein expression becomes more intense as tumor progresses, suggesting that post-translational modifications may play a major role in this molecule, possibly linking it to the loss of cell architecture, as observed in Figure 2. Additionally, *CK18* transcripts presented significant higher levels in pT3 tissues compared to other stages (Figure 2A). It was not verified modifications in ANXA1/CK18 ratio in benign tissues, with a concert expression of both markers. However, during cancer development and progression this proportion was altered, even in lower tumor staging.

Analysis of the concomitant expression and distribution of ANXA1 and CK18 in benign tissues demonstrated co-localization of both in ductal and glandular epithelial cells (Figure 2B). However, during malignant transformation, the ANXA1 expression in BC tissues was reduced in the malignant epithelium, but

retained in the myoepithelium. Other stromal cells such as fibroblasts were also positive. Furthermore, the antibody against CK18 reacted with epithelial cells in both benign and tumor tissues, but with increasing intensities as tumor progresses (Figure 2 B-F).

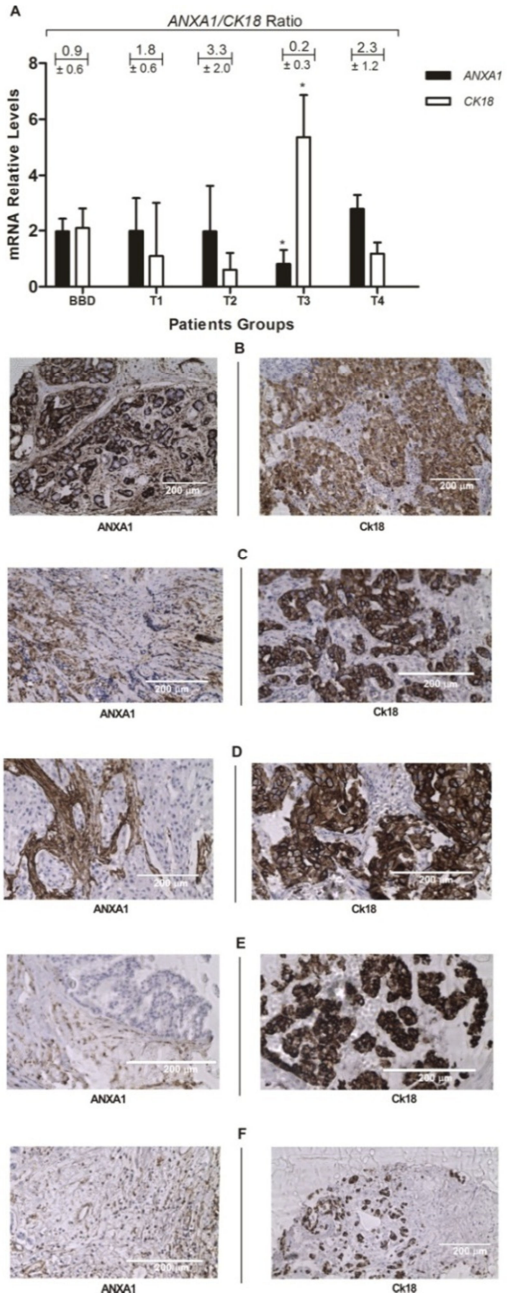

Figure 2: Transcriptional quantification of *ANXA1* and *CK18* and protein expression in benign samples and according to TNM system. In **(A)** we present relative mRNA levels of *ANXA1* and *CK18* . The values above the bars represent ANXA1/CK18 ratio for each histopathological classification and standard deviation (±). The transcripts were significantly different in pT3 classification comparing to all others stages. Immunohistochemical staining for annexin A1 (ANXA1) and cytokeratin 18 (CK18) in epithelial cells from BBD **(B)** and in breast tumor cells classified as pT1**(C)**; pT2 **(D)**; pT3 **(E)** and pT4 **(F)**. BBD, benign breast disease; BC, breast cancer. (*) P<0.05

Considering BC molecular subtypes (Figure 3), *ANXA1* and *CK18* transcript levels presented similar behavior, although with greater ratio discrepancies in triple-negative BCs. However, the ANXA1 and CK18 expression in tissues were differentially expressed and distributed across molecular subtypes, with greater differences in the basal-like phenotype, in which ANXA1 was highly expressed, while CK18 was down-regulated, showing an epithelial-mesenchymal transition (Figure 3D).

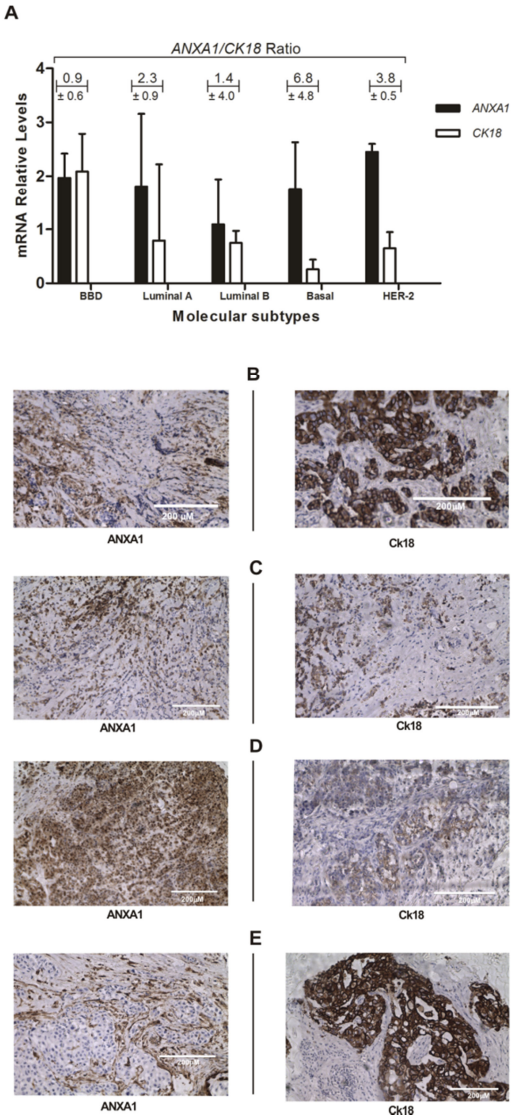

Figure 3: Transcriptional and protein expression of ANXA1 and CK18 in breast molecular subtypes. The relative mRNA levels are presented in **(A)** and immunostaining for ANXA1 and CK18 breast cancer tissues classified according to molecular subtypes characterization in: Luminal A **(B)**; Luminal B **(C)**; Triple-Negative **(D)** and Her-2 overexpression **(E)**. *CK18* transcripts were significantly lower in Her-2 tumors compared to other molecular subtypes. (*) P<0.05; TNBC: triple-negative breast cancer.

Alteration in *ANXA1*/*CK18* ratio also characterized the tumor progression and cell differentiation (Figure 4). Transcriptional levels of *CK18* were higher in benign tissues compared to breast cancer samples and it was observed a downregulation of *ANXA1* transcripts according to histological grade (Figure 4A). CK18 protein staining was lower in epithelial cells as tumor progresses and ANXA1 immunostainning was higher in myoepithelial cells in G3 (Figure 4 C-E).

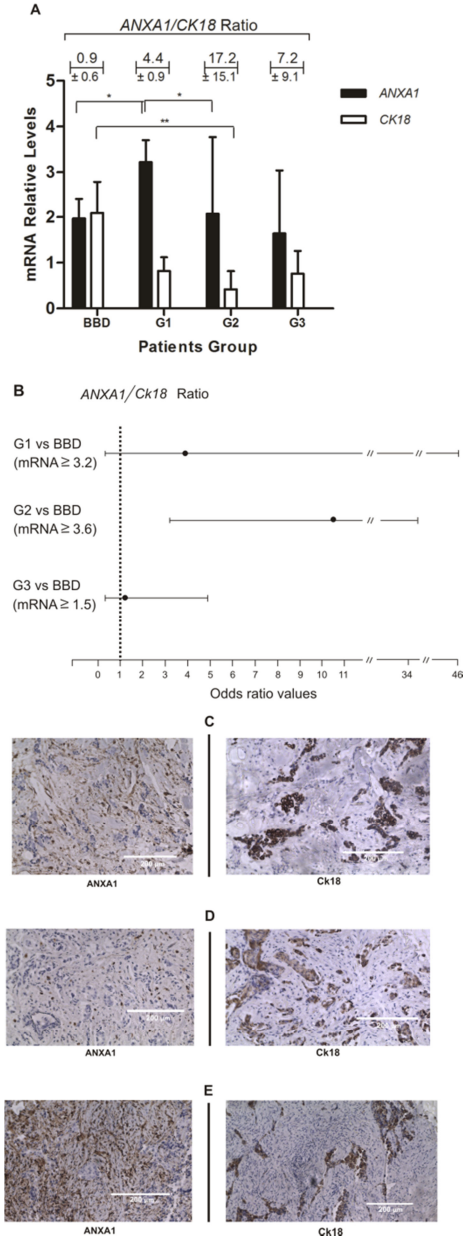

Figure 4: Relative quantification of messenger RNA (mRNA) and immunohistochemistry of ANXA1 and CK18 according to tumor grade. In **(A)** we present transcripts levels of *ANXA1* and *CK18* in benign, grade 1(G1), grade 2 (G2), and grade 3 (G3) tissues. *ANXA/CK18* ratio is annotated above the bars, with standard deviation (±) and in **(B)** is demonstrated the odds ratio for *ANXA/CK18* ratio calculated for Histhological grade vs Benign Breast Disease. Breast cancer tissues classified as G1, G2, and G2 and staining against ANXA1 and CK18 antibodies are represented in **(C)**, **(D)** and **(E)**, respectively. (*) P<0.05 and (**) P<0.005.

DISCUSSION

The cell core machinery for malignant cell transformation and development of breast cancer is intrinsically associated with unbalance between proliferation and apoptosis[21], with an intimate link between cell architecture and signaling. In our study, two important molecules deeply involved in these two processes, and also highly associated with breast cancer, *ANXA1* and *CK18*, were found to be tightly co-regulated in order to maintain the normal architecture and signaling of breast tissues, but changes in their expression and tissue distribution characterized tumor development and progression. These two molecules may be playing multifaceted roles in breast cancer development, progression, and metastasis, and the evidences of an important interaction for tumor initiation and progression are presented in this study.

Differential expression of *ANXA1* in human cancers has been reviewed elsewhere[22], generally showing reduced protein levels in head and neck cancer, esophageal and prostate cancer, and in B-cell non-Hodgkin's lymphoma. However, temporal and spatial changes in expression of *ANXA1* in breast tumors are not well defined and still controversial. *ANXA1* expression in ductal cells from both primary breast cancer tissue and breast cancer with lung metastases are shown to be increased when compared to ductal luminal cells from normal breast tissue[23]. On the other hand, reduced *ANXA1* expression has also been observed in both ductal carcinoma in situ (DCIS) and invasive breast cancer tissue[24]. In our investigation, we did not detect *ANXA1* expression differences in either cancer types or stages, except in pT3, which was significantly reduced, probably indicating a set point for metastasis [25].

Our interest in this joint analysis with *CK18* relies on the fact that *ANXA1* is a specific ligand of both *CK8* and *CK18*[3]. These intermediate filaments (cytokeratins) have been used as molecular markers in diagnostic and an aberrant expression of individual CKs results in abnormal cell behavior[26]. We have shown that *CK18* transcript levels were able to distinguish between benign and tumors tissues, and its downregulation was generally correlated with breast cancer development [27].

An important and significant disequilibrium between *ANXA1* and *CK18* expression and distribution was observed in all tumor stages, and may be partially

explained by differential phosphorylation at serine residues of *ANXA1*[28], which leads to loss of its apoptotic role [29] preserving the actin cytoskeleton, and inducing cell proliferation [30].

Interestingly, *CK8*/*CK18* complex assembly is also modulated by serine phosphorylation[31], although *CK8* can also be modulated by tyrosine phosphorylation, but not *CK18*[32], suggesting a connected action between *CK18* and *ANXA1* by serine phosphorylation. Serine phosphorylation of *ANXA1* has also been linked to pituitary adenomas, but not tyrosine phosphorylation[33], which may also be implicated in its tissue redistribution in other malignant tumors.

The actin cytoskeleton reorganization and its link with *CK18* and tumor progression can be better understood at the sentinel lymph node (SLN), the first lymph node that receives drainage from a primary tumor that is constantly loaded with lymphatic endothelial cells, resulting in high levels of shear stress, which may contribute to the production of a suitable environment for pre-metastatic environment[34]. As a consequence of the shear stress induction, a dramatic reduction of the soluble keratin component is observed followed by transformation of fine bundles of keratin IFs into thicker tonofibrils, and both effects are accompanied by the disappearance of most keratin particles and by increased phosphorylation of *CK8* and *CK18* on serine residues 73 and 33, respectively[35]. This is corroborated by our IHC data and transcriptional profiles of *CK18* across disease stages, which is constantly reduced during BC progression, except at the pT3 stage, with a profound difference in *ANXA1*:*CK18* ratios in cancer tissues, when homeostasis with equal levels of transcription was the expected pattern in benign tissues, suggesting that *ANXA1* signaling is compromised.

Considering *ANXA1* transcript levels across tumor stages and BBD, we have shown a consistent expression pattern, and besides a small reduction in pT3, its expression alone did not explain tumor development. However, its tissue distribution and expression are not correlated with their mRNA levels, and showed not only differential protein expression, but also presented a redistribution from epithelial to stromal compartments.

Our data for basal-like subtypes and pT3 and pT4 expression patterns suggest a switch of expression pattern of *CK18* and *ANXA1*, with a bimodal behavior that seems to be essential for an invasive phenotype. The ratio change

favored the *CK18* higher expression observed in the pT3 stage, which seems to be a key molecular event for tissue remodeling and might be linked to the highest shear stress level at the SLN, consequently with a probable association with tumor extravasation and dissemination. On the other hand, high *CK18* expression in tumor cells has been associated with reduced invasiveness *in vitro* or weakly metastatic cell line[36-38], and downregulation of CK18 has been demonstrated as a common and clinically relevant event in breast cancer [27]. Observations of a *CK18*-transfected invasive cell line demonstrated at least partial redifferentiation of the tumor cell[36]; however, the balance with *ANXA1* expression was not evaluated, and it is possible that the transfection has brought the *CK18* levels to similar expression levels of *ANXA1*, leading to the regression of the malignant phenotype. Our data support the notion that *CK18* expression variation is part of the modulation required for cell architecture reorganization and association with other proteins for signaling, such as *ANXA1*. During cancer development, specifically at pT1 and pT2 stages, *CK18* expression was downregulated, suggesting a dysfunctional cytoskeleton assembly without a concerted signaling at the glandular epithelial cell level, which is one of the first BC events, a profile that was maintained at pT4, after the *CK18* expression switch at the pT3. However, this instant changes in the *CK18* expression during malignant progression may be critical to EMT switch and to tumor cells to become mobile and invasive. The molecular profile of CK18 may be an essential event that directly induces shear stress and dissemination. On the other hand, the altered expression ratio (ANXA1/CK18) in pT3 may also be indirectly associated with invasiveness and dissemination through a positive feedback of *CK18* in pT3, possibly due to its cleavage or partial degradation [39], which induces an upregulation of its transcriptional levels. This is also corroborated by the reduced expression of ANXA1 in pT3 that is associated with EMT and metastasis [25]. Probably this could be the link between the post-translational processing and its transcriptional control. The levels of both markers observed in pT4 seems to follow the patterns observed in pT1 and pT2, which may be required for the establishment and proliferation of the cancer cells, but now in distant sites. This is also corroborated by the association of loss of expression of CK18 with micrometastatic cancer cell lines and higher grade tumors [40].

Annexins function as organizers of membrane domains and membrane recruitment platforms for proteins with which they interact, enabling them to participate in events that range from membrane dynamics to cell differentiation and migration[41], and this may explain the importance of the connection between *ANXA1* and *CK18* in BC. This intimate connection between *ANXA1* and *CK18* has been demonstrated by their co-localization in normal epithelial breast tissue, with a striking dichotomous redistribution during cancer development. *ANXA1* was found in the stromal of tumor tissues, the *CK18* was mainly found in the epithelium. Therefore, different from tissue homeostasis and mechanical equilibrium in normal physiological conditions, the disrupted tissue structure and organization during tumor evolution has led to unbalanced physical forces and altered properties of tumor components[42], which is evidenced in this study by a disequilibrium between *CK18* and *ANXA1*.

In brief, we showed that benign tissues present a homogeneous distribution of both *CK18* and *ANXA1* in the ducts with similar transcript levels, but during cancer development and progression this stable transcription is lost, which is aggravated by a dysfunctional post-translational modification that led to a dramatic dichotomous protein distribution in tumor tissues. This is the first description of the concomitant protein distribution of both *ANXA1* and *CK18* in cancer showing a surprising discrepancy in their distribution with different localizations, which is further corroborated by the significant alterations in their transcriptional expression ratios during cancer progression (*ANXA1*:*CK18*), supporting the notion that a homeostatic action of both molecules are necessary to maintain cellular architecture and correct signaling, and modifications may lead to tumor development. The combined use of both molecules in a reverse transcription-qPCR assay has proven to be an important diagnostic strategy, and could be as an auxiliary tool for disease diagnostics and staging.

Acknowledgements

The authors would like to thank the patients and their families for the direct collaboration in this work, the medical staff from the Gynecology Division of the University Hospital for providing the biological samples and the clinical parameters, and the financial support by CNPq, CAPES, and FAPEMIG.

Declaration of Interest

We confirm that all authors fulfill all conditions required for authorship. We also confirm that there is no potential conflict of interest as described in the Instruction for Authors. All authors have read and approved the manuscript.

REFERENCES

1. Pallari HM, Eriksson JE (2006) Intermediate filaments as signaling platforms. Sci STKE 2006 (366):pe53. doi:10.1126/stke.3662006pe53

2. Ditzel HJ, Strik MC, Larsen MK, Willis AC, Waseem A, Kejling K, Jensenius JC (2002) Cancer-associated cleavage of cytokeratin 8/18 heterotypic complexes exposes a neoepitope in human adenocarcinomas. J Biol Chem 277 (24):21712-21722. doi:10.1074/jbc.M202140200

3. Croxtall JD, Wu HL, Yang HY, Smith B, Sutton C, Chang BI, Shi GY, Flower R (1998) Lipocortin 1 co-associates with cytokeratins 8 and 18 in A549 cells via the N-terminal domain. Biochim Biophys Acta 1401 (1):39-51

4. Gerke V, Creutz CE, Moss SE (2005) Annexins: Linking Ca2+ signalling to membrane dynamics. Nature Reviews Molecular Cell Biology 6 (6):449-461

5. de Graauw M, van Miltenburg MH, Schmidt MK, Pont C, Lalai R, Kartopawiro J, Pardali E, Le Devedec SE, Smit VT, van der Wal A, Van't Veer LJ, Cleton-Jansen AM, ten Dijke P, van de Water B (2010) Annexin A1 regulates TGF-beta signaling and promotes metastasis formation of basal-like breast cancer cells. Proc Natl Acad Sci U S A 107 (14):6340-6345. doi:10.1073/pnas.0913360107

6. Cronstein BN, Molad Y, Reibman J, Balakhane E, Levin RI, Weissmann G (1995) Colchicine alters the quantitative and qualitative display of selectins on endothelial cells and neutrophils. J Clin Invest 96 (2):994-1002. doi:10.1172/JCI118147

7. Dustin P, Jr. (1963) New Aspects of the Pharmacology of Antimitotic Agents. Pharmacol Rev 15:449-480

8. Tiozzo R, Monti D, Straface E, Capri M, Croce MA, Rainaldi G, Franceschi C, Malorni W (1996) Antiproliferative activity of 3-aminobenzamide in A431 carcinoma cells is associated with a target effect on cytoskeleton. Biochem Biophys Res Commun 225 (3):826-832. doi:10.1006/bbrc.1996.1258

9. Miquel K, Pradines A, Favre G (1996) Farnesol and geranylgeraniol induce actin cytoskeleton disorganization and apoptosis in A549 lung adenocarcinoma cells. Biochem Biophys Res Commun 225 (3):869-876. doi:10.1006/bbrc.1996.1265
10. Flower RJ, Rothwell NJ (1994) Lipocortin-1: cellular mechanisms and clinical relevance. Trends Pharmacol Sci 15 (3):71-76
11. Perretti M, Gavins FN (2003) Annexin 1: an endogenous anti-inflammatory protein. News Physiol Sci 18:60-64
12. Solito E, Christian HC, Festa M, Mulla A, Tierney T, Flower RJ, Buckingham JC (2006) Post-translational modification plays an essential role in the translocation of annexin A1 from the cytoplasm to the cell surface. FASEB J 20 (9):1498-1500. doi:10.1096/fj.05-5319fje
13. Ciocca V, Bombonati A, Gatalica Z, Di Pasquale M, Milos A, Ruiz-Orrico A, Dreher D, Folch N, Monzon F, Santeusanio G, Perou CM, Bernard PS, Palazzo JP (2006) Cytokeratin profiles of male breast cancers. Histopathology 49 (4):365-370. doi:10.1111/j.1365-2559.2006.02519.x
14. Linder S, Havelka AM, Ueno T, Shoshan MC (2004) Determining tumor apoptosis and necrosis in patient serum using cytokeratin 18 as a biomarker. Cancer Letters 214 (1):1-9. doi:Doi 10.1016/J.Canlet.2004.06.032
15. Trask DK, Band V, Zajchowski DA, Yaswen P, Suh T, Sager R (1990) Keratins as Markers That Distinguish Normal and Tumor-Derived Mammary Epithelial-Cells. Proc Natl Acad Sci U S A 87 (6):2319-2323
16. Malzahn K, Mitze M, Thoenes M, Moll R (1998) Biological and prognostic significance of stratified epithelial cytokeratins in infiltrating ductal breast carcinomas. Virchows Archiv-an International Journal of Pathology 433 (2):119-129
17. Magin TM, Vijayaraj P, Leube RE (2007) Structural and regulatory functions of keratins. Experimental cell research 313 (10):2021-2032. doi:Doi 10.1016/J.Yexcr.2007.03.005
18. Shen DJ, Nooraie F, Elshimali Y, Lonsberry V, He JB, Bose S, Chia D, Seligson D, Chang HR, Goodglick L (2006) Decreased expression of annexin A1 is correlated with breast cancer development and progression as determined by a

tissue microarray analysis. Human Pathology 37 (12):1583-1591. doi:Doi 10.1016/J.Humpath.2006.06.001
19. Kakehashi A, Inoue M, Wei M, Fukushima S, Wanibuchi H (2009) Cytokeratin 8/18 overexpression and complex formation as an indicator of GST-P positive foci transformation into hepatocellular carcinomas. Toxicology and Applied Pharmacology 238 (1):71-79. doi:Doi 10.1016/J.Taap.2009.04.018
20. Ratkaj I, Stajduhar E, Vucinic S, Spaventi S, Bosnjak H, Pavelic K, Kraljevic Pavelic S (2010) Integrated gene networks in breast cancer development. Funct Integr Genomics 10 (1):11-19. doi:10.1007/s10142-010-0159-2
21. Parton M, Dowsett M, Smith I (2001) Studies of apoptosis in breast cancer. BMJ 322 (7301):1528-1532
22. Debret R, El Btaouri H, Duca L, Rahman I, Radke S, Haye B, Sallenave JM, Antonicelli F (2003) Annexin A1 processing is associated with caspase-dependent apoptosis in BZR cells. FEBS Lett 546 (2-3):195-202
23. Wallner BP, Mattaliano RJ, Hession C, Cate RL, Tizard R, Sinclair LK, Foeller C, Chow EP, Browing JL, Ramachandran KL, et al. (1986) Cloning and expression of human lipocortin, a phospholipase A2 inhibitor with potential anti-inflammatory activity. Nature 320 (6057):77-81. doi:10.1038/320077a0
24. Bruggers CS, Fults D, Perkins SL, Coffin CM, Carroll WL (1999) Coexpression of genes involved in apoptosis in central nervous system neoplasms. J Pediatr Hematol Oncol 21 (1):19-25
25. Maschler S, Gebeshuber CA, Wiedemann EM, Alacakaptan M, Schreiber M, Custic I, Beug H (2010) Annexin A1 attenuates EMT and metastatic potential in breast cancer. EMBO molecular medicine 2 (10):401-414. doi:10.1002/emmm.201000095
26. Willipinski-Stapelfeldt B, Riethdorf S, Assmann V, Woelfle U, Rau T, Sauter G, Heukeshoven J, Pantel K (2005) Changes in cytoskeletal protein composition indicative of an epithelial-mesenchymal transition in human micrometastatic and primary breast carcinoma cells. Clinical Cancer Research 11 (22):8006-8014. doi:Doi 10.1158/1078-0432.Ccr-05-0632
27. Woelfle U, Sauter G, Santjer S, Brakenhoff R, Pantel K (2004) Down-regulated expression of cytokeratin 18 promotes progression of human breast cancer.

Clinical cancer research : an official journal of the American Association for Cancer Research 10 (8):2670-2674
28. Dorovkov MV, Kostyukova AS, Ryazanov AG (2011) Phosphorylation of annexin A1 by TRPM7 kinase: a switch regulating the induction of an alpha-helix. Biochemistry 50 (12):2187-2193. doi:10.1021/bi101963h
29. Perretti M, Solito E (2004) Annexin 1 and neutrophil apoptosis. Biochem Soc Trans 32 (Pt3):507-510. doi:10.1042/BST0320507
30. Alldridge LC, Bryant CE (2003) Annexin 1 regulates cell proliferation by disruption of cell morphology and inhibition of cyclin D1 expression through sustained activation of the ERK1/2 MAPK signal. Experimental cell research 290 (1):93-107
31. Toivola DM, Omary MB, Ku NO, Peltola O, Baribault H, Eriksson JE (1998) Protein phosphatase inhibition in normal and keratin 8/18 assembly-incompetent mouse strains supports a functional role of keratin intermediate filaments in preserving hepatocyte integrity. Hepatology 28 (1):116-128. doi:10.1002/hep.510280117
32. Feng L, Zhou X, Liao J, Omary MB (1999) Pervanadate-mediated tyrosine phosphorylation of keratins 8 and 19 via a p38 mitogen-activated protein kinase-dependent pathway. J Cell Sci 112 (Pt 13):2081-2090
33. Mulla A, Christian HC, Solito E, Mendoza N, Morris JF, Buckingham JC (2004) Expression, subcellular localization and phosphorylation status of annexins 1 and 5 in human pituitary adenomas and a growth hormone-secreting carcinoma. Clin Endocrinol (Oxf) 60 (1):107-119
34. Kawai Y, Kaidoh M, Yokoyama Y, Ohhashi T (2012) Pivotal roles of shear stress in the microenvironmental changes that occur within sentinel lymph nodes. Cancer Sci. doi:10.1111/j.1349-7006.2012.02289.x
35. Flitney EW, Kuczmarski ER, Adam SA, Goldman RD (2009) Insights into the mechanical properties of epithelial cells: the effects of shear stress on the assembly and remodeling of keratin intermediate filaments. FASEB J 23 (7):2110-2119. doi:10.1096/fj.08-124453
36. Buhler H, Schaller G (2005) Transfection of keratin 18 gene in human breast cancer cells causes induction of adhesion proteins and dramatic regression of malignancy in vitro and in vivo. Molecular Cancer Research 3 (7):365-371

37. Schaller G, Fuchs I, Pritze W, Ebert A, Herbst H, Pantel K, Weitzel H, Lengyel E (1996) Elevated keratin 18 protein expression indicates a favorable prognosis in patients with breast cancer. Clinical Cancer Research 2 (11):1879-1885
38. Weng YR, Cui Y, Fang JY (2012) Biological Functions of Cytokeratin 18 in Cancer. Molecular Cancer Research 10 (4):485-493. doi:Doi 10.1158/1541-7786.Mcr-11-0222
39. Omary MB, Ku NO, Tao GZ, Toivola DM, Liao J (2006) "Heads and tails" of intermediate filament phosphorylation: multiple sites and functional insights. Trends in biochemical sciences 31 (7):383-394. doi:10.1016/j.tibs.2006.05.008
40. Willipinski-Stapelfeldt B, Riethdorf S, Assmann V, Woelfle U, Rau T, Sauter G, Heukeshoven J, Pantel K (2005) Changes in cytoskeletal protein composition indicative of an epithelial-mesenchymal transition in human micrometastatic and primary breast carcinoma cells. Clinical cancer research : an official journal of the American Association for Cancer Research 11 (22):8006-8014. doi:10.1158/1078-0432.CCR-05-0632
41. Gerke V, Creutz CE, Moss SE (2005) Annexins: linking Ca2+ signalling to membrane dynamics. Nat Rev Mol Cell Biol 6 (6):449-461. doi:10.1038/nrm1661
42. Yu H, Mouw JK, Weaver VM (2011) Forcing form and function: biomechanical regulation of tumor evolution. Trends Cell Biol 21 (1):47-56. doi:10.1016/j.tcb.2010.08.015

CAPÍTULO 4

Análise do perfil transcricional de citoqueratinas como preditor dos subtipos moleculares de Câncer de Mama

[Capítulo escrito de acordo com as normas exigidas pela revista Clinical Chemistry and Laboratory Medicine]

Title: Cytokeratins' transcripts as predictors of breast cancer molecular subtypes

Autorship

Thaise Gonçalves de Araújo[1], Karina Marangoni[1], Galber Rodrigues Araújo[1], Yara Cristina de Paiva Maia[1], Patrícia Terra Alves[1], Carlos Ueira Vieira[1], Luiz Ricardo Goulart[1,2]

Affiliation

[1]Laboratory of Nanobiotechnology, Institute of Genetics and Biochemistry, Federal University of Uberlandia, Uberlandia, Minas Gerais, Brazil.

[2]University of California Davis, Dept. of Medical Microbiology and Immunology, Davis, CA, USA.

TGA: thaise_araujo@yahoo.com.br

KM: kmarangoni@yahoo.com.br

GRA: galber.araujo@gmail.com

YCPM: yarapaivamaia@gmail.com

PTA: patriciaterraalves@yahoo.com.br

CUV: ueirabio@gmail.com

LRG: lrgoulart@ufu.br

Corresponding author: Thaise Gonçalves de Araújo, Laboratory of Nanobiotechnology, Institute of Genetics and Biochemistry, Federal University of Uberlandia, Campus Umuarama, Bloco 2E, Sala 248, 38400-902, Uberlandia, MG, Brazil. Phone: +55 34 3218 2478. thaisegaraujo@gmail.com

RESUMO

As citoqueratinas (CK) pertencem a uma grande família de filamentos intermediários que estão primariamente expressas em células epiteliais e cujos membros possuem diferentes comportamentos nas doenças malignas e benignas. No Câncer de Mama (CM), a caracterização das citoqueratinas é uma ferramenta importante no histodiagnóstico oferecendo parâmetros moleculares na avaliação

do perfil clínico das pacientes. Neste estudo, nós avaliamos os níveis de RNA mensageiro das citoqueratinas *CK5, CK6, CK8, CK14* e *CK18* por qPCR e verificamos que os transcritos *CK18*, *CK5* e *CK14* foram efetivos na diferenciação entre tumores malignos mamários e doenças benignas da mama. A quantificação relativa dos níveis transcricionais da *CK18* foram 2.08 vezes maior nas doenças benignas da mama do que em CM. Foi observado um mesmo comportamento na expressão de *CK5* e *CK14*, os quais foram 2.4 e 4.8-vezes maiores em tecidos não malignos e a *CK18* se correlacionou com os níveis de expressão do mRNA das *CK5*, *CK6*, *CK8* e *CK14*. Considerando os subtipos moleculares as citoqueratinas 8/18 e 5/14 apresentaram o mesmo comportamento em seus níveis de mRNA . Além disso, a *CK18* se correlacionou ao fenótipo clinico dos tumores. Nossos resultados sugerem, portanto, que os mecanismos envolvidos com o aumento de expressão de citoqueratinas pode ser conservados dentre seus diferentes tipos e que os transcritos de *CK18*, *CK5* e *CK14* podem desempenhar um importante papel na gênese da doença diferenciando células tumorais malignas e benignas.

Palavras chave: citoqueratinas, câncer de mama, níveis transcricionais, qPCR

ABSTRACT

Cytokeratins (CK) belong to a large family of intermediate filaments that are primarily expressed in epithelial cells and whose members are in various combinations in normal and malignant entities. In Breast Cancer (BC), keratin typing is a major tool in tumor histodiagnosis providing molecular parameters to assess its differentiation status. In our study we evaluated *CK5*, *CK6*, *CK8*, *CK14* and *CK18* mRNA levels by qPCR, and *CK18*, *CK5* and *CK14* transcripts were effective in differentiate breast tumors and benign diseases. Relative quantification of *CK18* mRNA levels was 2.08-fold higher in benign breast disease than in BC. It was observed the same expression behavior for *CK5* and *CK14*, which were 2.4 and 4.8-fold higher in nonmalignant tissues, and *CK18* correlated with *CK5*, *CK6*, *CK8* and *CK14* mRNA expression. Cytokeratins 8/18 and 5/14 presented the same behavior according to molecular subtypes. However, *CK18* itself correlated to clinical outcome in tumors phenotyping. We suggest that the mechanisms underlying the increased expression of cytokeratins proteins in breast tumors may

be similar and *CK18*, *CK5* and *CK14* transcripts may be important in tumorigenic process, distinguishing normal from tumor cells.

.

Keywords: cytokeratins, breast cancer, mRNA expression levels, molecular technology, qPCR

INTRODUCTION

Several efforts have been devoted to identifying the molecular abnormalities contributing to breast cancer (BC) development and progress [(1)]. This malignant tumor is no longer seen as a single entity and patients with the same clinic-pathological parameters can have markedly different clinical courses [(2)]. Gene expression arrays have been able to genetically profile breast cancer into four distinct molecular subtypes: basal-like, luminal A and B; and erbB2/human epidermal growth factor receptor-2 (HER-2) oncogene status. Importantly, this molecular taxonomy has critical clinical value because some of these phenotypes show unfavorable prognosis and/or resistance to treatment. However, several factors are intertwined during malignant transformation and tumor development [(3,4)]. For this reason the search for molecular changes that may affect the biology of cancer development and progression may be important in understanding this disease and improve clinical management of BC patients [(5)].

Distinct keratins emerge as highly dynamic scaffolds involved in different settings and contribute to cell size determination, translation control, proliferation, cell type-specific organelle transport and malignant transformation [(6)]. In the mammary gland, cytokeratins (CK) expression is tightly regulated and correlates with the origin of the cells in the ducts [(7)]. In the bilaminar breast epithelium, CK8 and CK18 characterize the differentiation compartment, whereas CK5 and CK14 are expressed in the proliferation compartment [(8)]. Expression of CK7, CK17 and CK19 is variable but generally low [(9-10)]. All of the CKs share the same domain structure and form heteropolymers [(11)], which have been recognized for more than 20 years as epithelial markers in diagnostic histopathology, where basal like cells express CK5, 6 and 14 and /or 17 (basal/myoepithelial phenotype) and luminal-like express CK8,18 and 19 [(12,13)]. A key observation of many studies is that keratin expression changes rapidly during differentiation, tissue injury and metastasis [(6)].

Breast tumorigenesis involves altered expression of proteins and transcripts, which can play an important role in cancer progression. The different biological behaviors and metastatic patterns observed among the distinct breast cancer phenotypes may suggest different mechanisms of invasion and metastasis for breast tumors.

It is becoming evident that a cell changes its expression patterns while it is progressing from a normal to an invasive panel and tumor growth is not just a result of uncontrolled proliferation but also of reduced apoptosis, which involve regulation of cytokeratins. Therefore, elucidating new molecular associations may provide clues to novel diagnostic, prognostic and therapeutic approaches to treat BC [14-15].

Due to the heterogeneous, multifactorial and multifocal nature of breast cancer, the search for potential biomarkers and its molecular associations involved in the occurrence and development of this disease is fundamental for a more precise diagnosis and to clarify the neoplastic phenotype. Considering that most cancers also changes its keratins profile we aimed clarifying the relevance of cytokeratins 5,6, 8,14 and 18 mRNA in human BC, assessed by quantitative RT-PCR demonstrating possible molecular pathway that is associated with this tumor occurrence and characterization.

MATERIALS AND METHODS

Study design and sample collection

This Project was carried out from 2010 to 2011 at the Nanobiotechnology Laboratory of the Federal University of Uberlandia (UFU) together with the Obstetric Service of University Hospital. The study protocol was approved under the number 176/2008 by the local Research Ethics Committee in accordance with the Helsinki Declaration of 1975, as revised in 2008, and an informed consent was obtained from all the participants.

The breast samples encompass materials from 80 patients grouped in two classes: 40 BC, and 40 benign breast diseases (BBD). Classification of patients was made according to clinical parameters. The average age of the patients investigated was 46.2 years (range 30-80 years) for BC group, and 46.8 years

(range 18-58 years) for BBD group. There were 15 (37.5%), 18 (45%), 3 (7.5%), and 4 (10%) breast tumors classified as TNM stages T_1, T_2, T_3 and T_4, respectively. The histological grading according to the Nottingham system were grade I (GI) in 4 (10%), GII 22 (55%), and GIII in 14 (35%).

Hormone receptors, estrogen receptor (ER) was positive in 29 (72.5%), negative in 6 (15%) and not evaluated in 5 (12.5%); progesterone receptor (PgR) was positive in 23 (57.5%), negative in 12 (30%) and not analyzed in 5 (12.5%). HER2 status was considered as positive (score 3+) and negative (score 0–1+); scores 2+ were excluded from the analyses. HER-2 status were positive in 9 cases (22.5%), negative in 25 (62.5%) and score 2+ or not analyzed in other 6 (15%).

In BC cases, surgical procedures were radical mastectomy and quadrantectomy, depending on the size of the tumor, with axillary dissection and were collected from untreated patients.

The mRNA analysis was carried out on fresh breast samples containing 50-80% of malignant portion. BC patients were characterized by their TNM staging, Scarff-Bloom-Richardson (SBR) grading, lymph node, hormone status, and Her2.

Quantitative RT-PCR

The mRNA was extracted in duplicates from fresh tumor and BBD breast tissues of each patient using the *Trizol* reagent (Invitrogen - Carlsbad, CA, USA) according to the manufacturer's recommendations. RNA extractions were carried out as described elsewhere [16].

Synthesis of cDNA was performed from 1µg of total mRNA at a total volume of 20µL containing 2U of *Murine Moloney Leukemia Virus* Reverse Transcriptase (MMLV-RT), 1X MMLV-RT Buffer, 0.1M of DTT, 1U of RNase inhibitor, 200µM of each dNTPs (desoxyribonucleotide) and 6µM of hexamer random primers (Invitrogen - Carlsbad, CA, USA). The reactions were incubated at 37ºC for 1h and heated at 95ºC for 5 min. For normalization of amplification reactions, an internal positive control gene was chosen, the constitutive β2-microglubuin (*B2M*) gene (Table 1), which was also used to validate reactions and to further characterize RNA quality of each sample.

Real-time PCR for *CK18*, *CK5*, *CK6*, *CK8*, *CK14* and *B2M* genes was performed using the ABI PRISM 7300 Sequence Detection System (Applied Biosystems - Carlsbad, CA, USA)). Sequence of primers used, annealing and detection temperatures are presented in Table 1. All primers were designed to not amplify genomic DNA (usually one is positioned on exon-exon junction). The PCR was conducted in a total volume of 10µL containing Power SYBR_ Green PCR Master Mix (Applied Biosystems - Carlsbad, CA, USA), 2µL cDNA (1:4) and 5µM of each primer. Dissociation and standard curves for all primers were constructed. PCR efficiency (E) was calculated according to the equation: $E = (10^{-1/slope} - 1) \times 100$ to validate the relative quantification based on comparative CT method. To compare levels of target genes mRNA between BBD patients and BC patients, one of BBD tissue samples was chosen as the calibrator sample to which all of the others was compared.

Table 1: Oligonucleotides sequences used for the multiple mRNA markers study.

Primer	GenBank acess	Sequence (forward/reverse)	Nucleotide position	Annealing temperature (ºC)	Amplified Product
KRT5	NM_000424	5'- AGATGTTCTTTGATGCGGAGC-3' 5'-CTGCGGTTGGCAATCTCCT-3'	1074-1094 1203-1221	60	148 bp
KRT6	NM_005554	5'-AGGGTGAGGAGTGCAGGCT-3' 5'-CCAAGACCACTGCCATAGGAG-3'	1618-1636 1754-1774	54	157 bp
KRT8	NM_002273	5'-AGCTGGAGTCTCGCCTGGA-3' 5'-CAGCTCCCGGATCTCCTCT-3'	729-747 790-808	60	80 bp
KRT14	NM_000526	5'-ATTGAGGACCTGAGGAACAAGATT-3' 5'-CGCAGGTTCAACTCTGTCTCAT-3'	566-589 672-693	60	128 bp
KRT18	NM_000224.2	5'-GCTCTGGGTTGACCGTGG-3' 5'-GTGGTGCTCTCCTCAATCTGC-3'	804-821 934-954	58	151 bp
B2M	NM_004048	5'-CCTGCCGTGTGAACCATGT-3' 5'-GCGGCATCTTCAAACCTCC-3'	356-374 449-431	54-60	94 bp

Data analysis

The non-parametric Mann-Whitney test was used for mean comparisons of the relative mRNA expression of the *CK18*, *CK5*, *CK6*, *CK8*, and *CK14* transcripts between BC and BBD patients. Spearman's correlation analysis was performed with all clinical parameters: patient age at diagnosis, TNM system staging, Scarff-Bloom-Richardson (SBR) grading and hormonal status, in which HER2 status was considered as positive (score 3+) and negative (score 0 / 1+); estrogen receptor (ER) and progesterone receptor (PR) was considered as positive (score 2+, 3+ and 4+) and negative (score 0 and 1+). Histogical grade of 2 and 3 were considered as high and 1 as low. A detection limit was established to calculate odds ratios for all significant genes expression and their combinations.

The Kendall's rank correlation (τ) was applied to determine the strength of association between the categorical variables. Statistical significance was considered when $P<0.05$. The statistical analyses were carried out using GraphPad Prism 5 (GraphPad Software Inc. - La Jolla, CA, USA) and SPSS version 17.0 (SPSS - Chicago, IL, USA) for Windows.

RESULTS

Analysis of cytokeratins gene expression levels

To elucidate cytokeratins crosstalk in mRNA levels we performed RT-PCR for all patients. The inter-individual variations of basal and luminal cytokeratins transcripts observed among patients are represented by medians and percentiles (25-75%) in Figure 1. Kendall's rank correlation demonstrated significant associations between *CK18* and *CK5*, *CK6*, *CK14* and *CK8* expression ($\tau = 0.51$, $\tau = 0.29$, $\tau = 0.50$ and $\tau = 0.29$). Lower *CK18* mRNA levels were detected in breast tumors compared with benign breast tissues ($P = 0.02$).

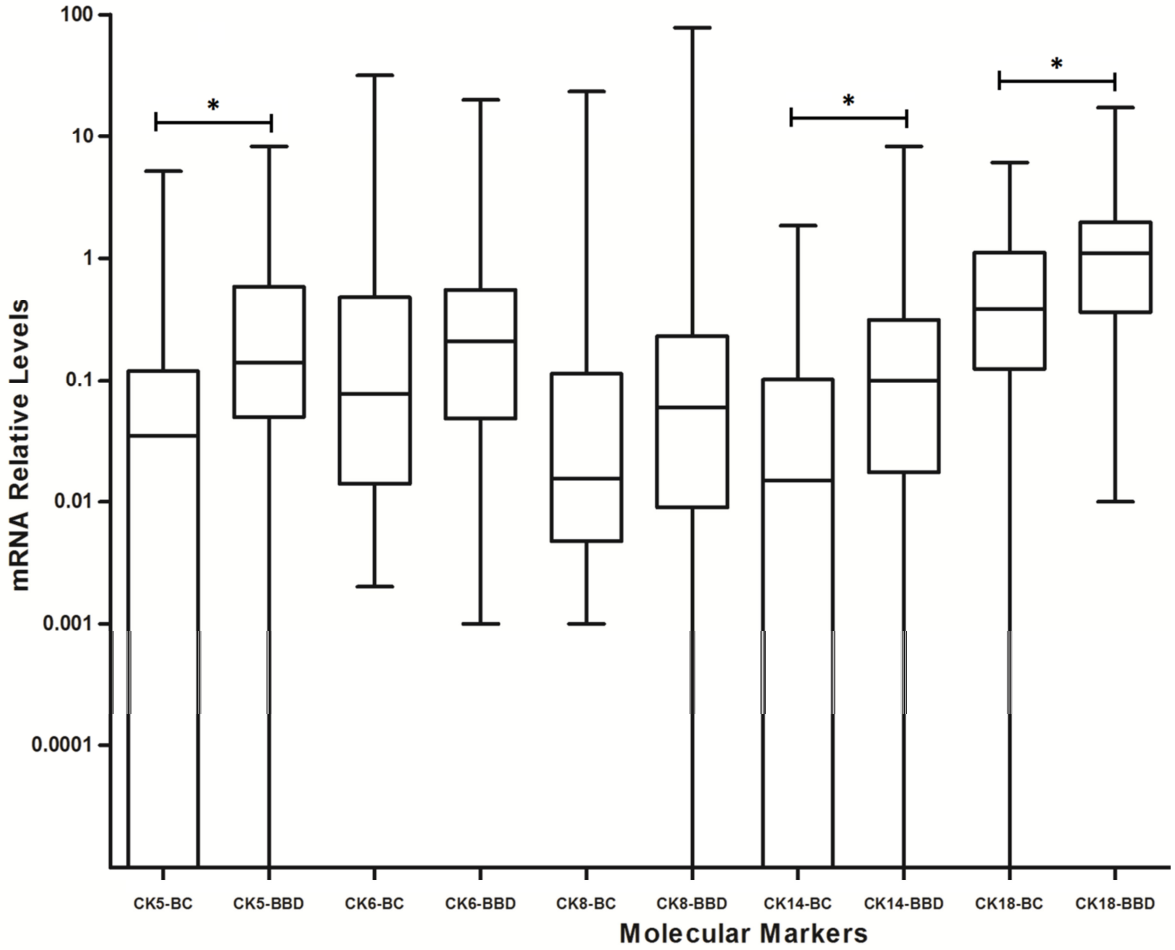

Figure 1: Medians and percentiles for relative *CK5*, *CK6*, *CK8*, *CK14*, *CK18* and *ANXA1* mRNA levels in benign and tumor breast tissues. (*) P<0.05

Relative quantification of *CK18* mRNA levels was 2.08-fold higher in BBD than in BC. It was observed the same expression behavior for *CK5* and *CK14*, which were 2.4 and 4.8-fold higher in nonmalignant tissues. Transcripts levels of *CK6* and *CK8* did not differed between these two study groups. A detection limit was estimated for *CK18*, *CK5* and *CK14* gene according to maximal differences between BC and BBD groups in their medians and percentiles. These limits were used to calculate the odds ratios values, estimated individually and combined for the targets genes (Table 2). As the cut-off value for the relative levels of transcript revealed a down-regulated expression of these cytokeratins in BC samples, the chance of having cancer was observed when the mRNA levels were lower than the detection limit: 0.02 for *CK5* and *CK14*, and 0.35 for *CK18.* A 4.89-higher

chance of having cancer was observed when *CK5* transcripts, alone, were lower than 0.02, which influenced the estimated values for the combined tests.

Table 2: Odds ratios estimated for *CK18*, *CK5* and *CK14* transcript levels in breast tissues. Detection limit was 0.02 for *CK5* and *CK14*, and 0.35 for *CK18*

Molecular markers	Positive RT-PCR		Odds Ratio (95% CI)
	BC patient no. (%)	BBD patient no. (%)	
CK5	17/39 (44)	6/44 (14)	4.894 (1.681 to 14.25) P = 0.0023
CK14	21/42 (50)	12/50 (24)	3.167 (1.304 to 7.691) P = 0.0096
CK18	20/41 (49)	11/50 (22)	3.377 (1.363 to 8.366) P = 0.0073
CK5/CK14	8/19 (42)	0/25 (0)	3.273 (2.000 to 5.356) P = 0.0003
CK5/CK18	7/19 (37)	0/26 (0)	3.167 (1.983 to 5.057) P = 0.0008
CK14/CK18	15/30 (50)	9/45 (75)	4.000 (1.438 to 11.12) P = 0.0064
CK18/CK5/CK14	6/14 (43)	0/24 (38)	4.000 (2.195 to 7.290) P = 0.0005

Cytokeratins and clinical outcomes

To elucidate the role of keratins as a prognostic tool we demonstrated the behavior of these molecular markers in tumor tissues, at mRNA levels, classifying samples according to their molecular subtypes. The average of cytokeratins levels are described in Figure 2A. Among Luminal patients, we detected higher transcripts of *CK18* and *CK8* compared to the others cytokeratins, as expected by the luminal cells behavior. Interestingly, *CK18* transcripts were able to discriminate the groups, including BBD patients. Considering hormonal parameters and Her2 separately we verified higher *CK18* transcripts in ER positive patients and a discrepancy with *CK8* mRNA levels in PR positive patients and Her-2 tumors. (Figure 1B-D). Additionally, considering positive PR patients, *CK18* presented

significantly higher transcripts compared to the others markers (Figure 1C). Considering molecular subtypes, *CK8/18* transcripts presented discrepancy as prognostic markers and *CK18* itself correlated to clinical outcome in tumors phenotyping.

CK5 expression, either in association with a triple negative status ore alone is often used to define the heterogeneous group of TNBC and basal-like breast tumors. However, there was no difference in average levels for *CK5* and *CK14* transcripts in TNBC group, only higher levels of *CK6* in negative HER-2 and PR patients (Figure 1C and D). Finally, in patients that over-express HER-2 was verified a higher expression of *CK18* and *CK8*, due to their behavior according to hormone status.

Analyzing pathological parameters, it was observed decreasing in *CK18*, *CK5* and *CK6* mRNA quantification in less differentiated tumors (Figure 1E) and lower expression of *CK18*, CK5 and *CK14* in negative lymph nodes (Figure 1F).

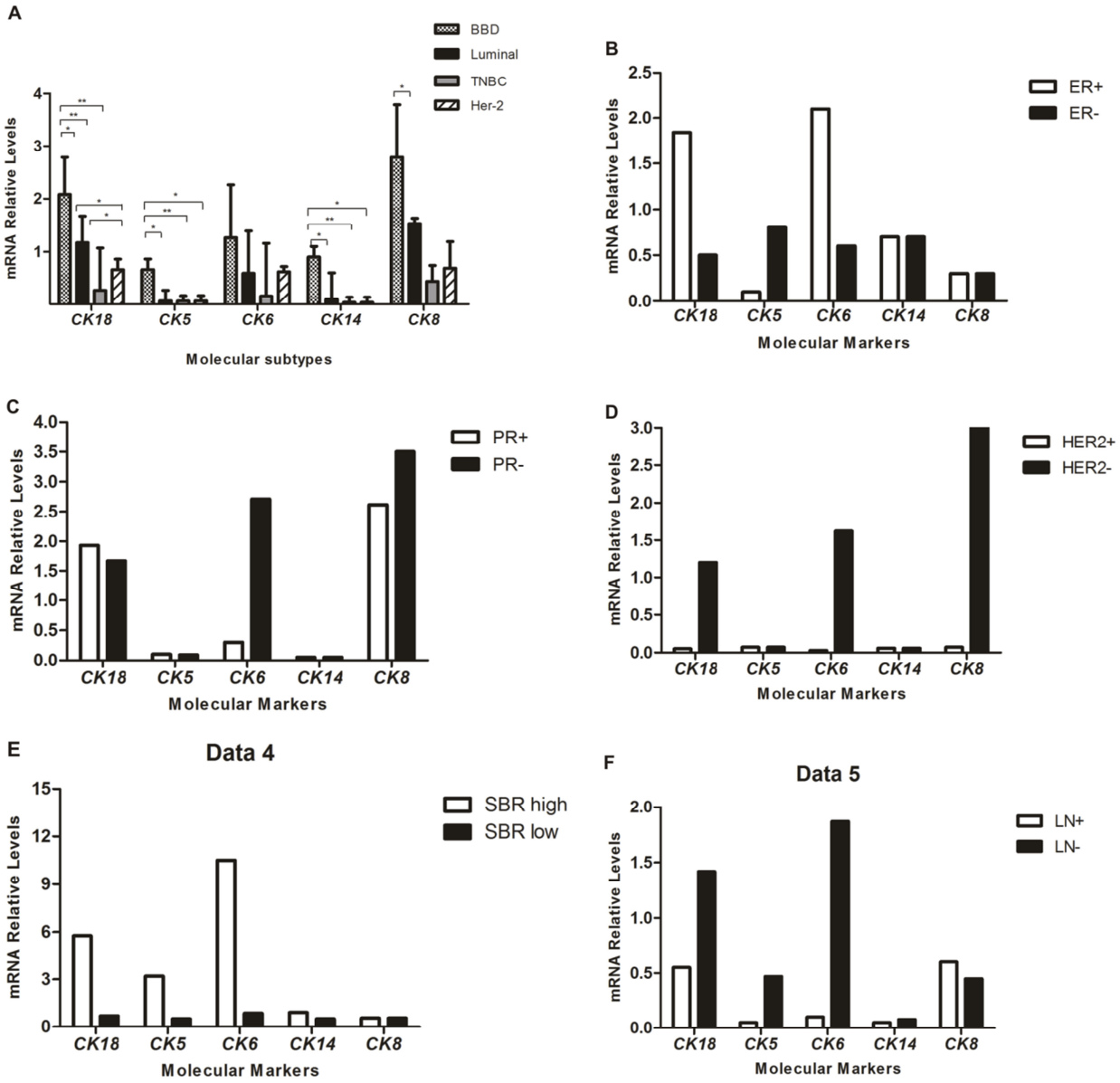

Figure 2: Cytokeratins profile in malignant tissues. Transcripts expression of *CK18, CK5, CK6, CK8* and *CK14* were characterized according to molecular subtypes: luminal-like, TNBC, and HER-2 overexpression **(A)**, hormone status **(B and C)**, HER-2 positivity **(D)**, Scarff-Bloom-Richardson (SBR) grading **(E)** and lymph nodes (LN) status **(F)**. Positive ER / PR: score 2+, 3+ and 4+; Negative ER / PR: score 0 and 1+. Positive HER-2; score 3+; Negative HER-2: score 0 and 1+. ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor-2; TNBC, Triple Negative Breast Cancer.

The average *CK18* levels presented a bimodal behavior classified according to tumor stages with higher levels in pT3 (Figure 3). However, the others cytokeratins demonstrated opposite behaviors with higher expression patterns in pT2 and pT4.

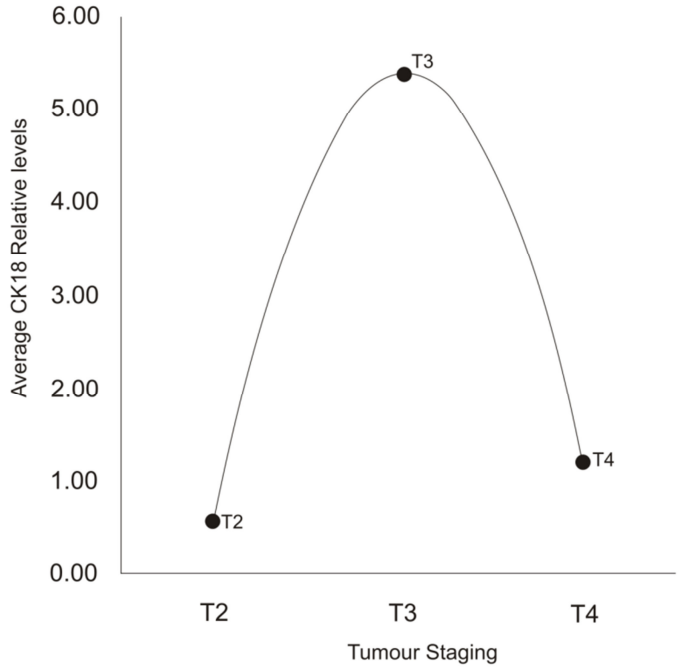

Figure 3: Graphic representation of average *CK18* levels of BC patients classified according to tumor stages and a predicted tendency line.

DISCUSSION

Breast cancer is an extremely complex and heterogeneous disease, both at the histological and molecular level. The accumulation of numerous and often unknown molecular alterations cause cell proliferation, genetic instability, and acquisition of an increasingly invasive and resistant phenotype. It is evident that a general hypothesis related the co-operation of multiple factors rather than involvement of a single factor is needed to induce malignancy is widely considered to be more likely to define therapeutic treatments directed at multiple molecular targets for more effective cancer treatment. Therefore, challenges in understanding the mechanisms leading to the appearance and progression of cancer must include monitoring proteins and profile gene expression signaling mechanisms [13].

We demonstrated higher expression of *CK18, CK5* and *CK14* mRNA in benign tissues compared to malignant samples. High CK18 expression in tumor cells has been associated with reduced invasiveness in vitro or weakly metastatic cell lines [8,17]. The down-regulation of *CK18* in pT1 and pT2 may indicate an important role of this marker as a putative suppressor of breast cancer progression. However, changes in composition of CK18 in the beginning of malignant progression may be critical to tumor cells become mobile and invasive.

For decades, intermediate filaments have been used as molecular markers in diagnostic and an aberrant expression of individual CKs results in abnormal cell behavior [18]. Correlation analysis of CK mRNA expression revealed a significant association to the expression of *CK18* with all others. This suggests that the mechanisms underlying the increased expression of cytokeratins proteins in breast tumors may be similar, possibly reflecting the events associated with tumor development and progression. *CK18*, *CK5* and *CK14* transcripts were able to distinguish benign from tumor patients and decreased of those expression correlated with breast cancer occurrence. This expression panel may indicate that the cells may be associated not only to proliferative function but also involving in perturbation by different signals from the microenvironment gives rise to benign diseases.

Among the identified CKs, elevated expression levels of *CK8* are known to be indicative of HER-2 positivity [4] and *CK14* presented higher expression in basal-like tumors. As expected, factor analysis showed that estrogen and progesterone receptors were the factor that most influenced *CK18* dataset. Different patterns in molecular subtypes indicate that the CK expression may vary during the natural course of the disease; an assumption supported by previous reports on down-regulation of CKs during breast cancer progression [12,19]. To date, the information derived from our understanding of the molecular features of breast cancer promises to provide additional diagnostic, prognostic and predictive information. In our study *CK5*, *CK14* and *CK18* transcripts may be useful to make the differential diagnosis between benign and malignant lesions. Importantly, such information may improve breast cancer management and, in the future, may facilitate the development of new therapy strategies.

Acknowledgements

The authors would like to thank the patients and his families for the direct collaboration in this work, the medical staff from the Gynecology Division of the University Hospital for providing the biological samples and the clinical parameters and the financial support by CNPq, CAPES and FAPEMIG.

Declaration of Interest

We confirm that all authors fulfill all conditions required for authorship. We also confirm that there is no potential conflict of interest, as described in the Instruction for Authors. All authors have read and approved the manuscript.

REFERENCES

1. Shen, D.; Nooraie, F.; Elshimali, Y.; Lonsberry, V.; He, J.; Bose, S.; Chia, D.; Seliqson, D.; Chang, H.R.; Goodglick, L. Decreased expression of annexin A1 is correlated with breast cancer development and progression as determined by a tissue microarray analysis. Hum Pathol **2006,** *37*, 1583-1591.

2. Paiva, C.E.; Drigo, S.A.; Rosa, F.E.; Moraes Neto, F.A.; Caldeira, J.R.F.; Soares, F.A.; Dominques, M.A.; Rogatto, S.R. Absence of transforming growth factor-β type II receptor is associated with poorer prognosis in HER2-negative breast tumours. Ann Oncol **2009**, *21*, 734-740.

3. Hartman, M.; Loy, E.Y.; Ku, C.S.; Chia, K.S. Molecular epidemiology and its current clinical use in cancer management. Lancet Oncol **2010**, *11*, 383-390.

4. Schulz, D.M.; Böllner, C.; Thomas, G.; Atkinson, M.; Esposito, I.; Höfler, H.; Aubele, M. Identification of differentially expressed proteins in triple-negative breast carcinomas using DIGE and mass spectrometry. J Proteome Res **2009**, *8*, 3430-3438.

5. Ahn, S.H.; Sawada, H.; Ro, J.Y.; Nicolson, G.L. Differential expression of annexin I in human mammary ductal epithelial cells in normal and benign and malignant breast tissues. Clin Exp Metastasis **1997**, *15*, 151-156.

6. Magin, T.M.; Vijayaraj, P.; Leube, R.E. Structural and regulatory functions of keratins. Exp Cell Res **2007**, *313*, 2021-2032.

7. Ciocca, V.; Bombonati, A.; Gatalica, Z.; Di Pasquale, M.; Milos, A.; Ruiz-Orrico, A.; Dreher, D.; Folch, N.; Monzon, F.; Santeusanio, G.; Perou, C.M.; Bernard,

P.S.; Palazzo, J.P. Cytokeratin profiles of male breast cancers. Histopathology **2006**, *49*, 365-370.

8. Bühler, H.; Schaller, G. Transfection of keratin 18 gene in human breast cancer cells causes induction of adhesion proteins and dramatic regression of malignancy in vitro and in vivo. Mol Cancer Res **2005**, *3*, 365-371.

9. Becker, M.; Nitsche, A.; Neumann, C.; Aumann, J.; Jonghahn, I.; Fichtner I. Sensitive PCR method for the detection and real-time quantification of human cells in xenotransplantation systems. Br J Cancer **2002**, *87*, 1328-1335.

10. Awadelkarim, K.D.; Arizzi, C.; Elamin, E.O.M.; Hama, H.M.A.; De Blasio, P.; Mekki, S.O.; Osman, I.; Biunno, I.; Elwali, N.E.; Barberis, M.C.; Mariani-Costantini,R . Basal-like phonotype in a breast carcinoma case series from Sudan: prevalence and clinical/pathological correlations. Patholog Res Int **2011**, *9*, 2011:806831.

11. Linder, S.L.; Havelka, A.M.; Ueno, T.; Shoshan, M.C. Determining tumor apoptosis and necrosis in patient serum using cytokeratin 18 as a biomarker. Cancer Lett **2004**, *214*, 1-9.

12. Trask, D.K.; Ban, V.; Zajchowski, D.A.; Yasmen, P.; Suh, T.; Sager, R. Keratins as markers that distinguish normal and tumor-derived mammary epithelial cells. Proc Natl Acad Sci USA **1990**, *87*, 2319-2323.

13. Malzahn, K.; Mitze, M.; Thoenes, M.; Moll, R. Biological and prognostic significance of stratified epithelial cytokeratins in infiltrating ductal breast carcinomas. Virchows Arch **1998**, *433*, 119-129.

14. Kakehashi, A.; Inoue, M.; Wei, M.; Fukushima, S.; Wanibuchi, H. Cytokeratin 8/18 overexpression and complex formation as an indicator of GST-P positive foci transformation into hepatocellular carcinomas. Toxicol Appl Pharmacol **2009**, *238*, 71-79.

15. Parton, M.; Dowsett, M.; Smith, I. Studies of apoptosis in breast cancer. BMJ **2001**, *322*, 1528-1532.

16. Neves, A.F.; Araújo, T.G.; Biase, W.K.F.S.; Meola, J.; Alcântara, T.M.; Freitas, D.G.; Goulart, L.R. Combined analysis of multiple mRNA markers by RT-PCR assay for prostate cancer diagnosis. Clin Biochem **2008**, *41*, 1191-1198.

17. Schaller, G.; Fuchs, I.; Pritze, W.; Ebert, A.; Herbst, H.; Pantel, K.; Weitzel, H.; Lengye, E. Elevated keratin 18 protein expression indicates a favorable prognosis in patients with breast cancer. Clin Cancer Res **1996**, *2*, 1879-1885.

18. Wilipinski-Stapelfeldt, B.; Riethdorf, S.; Assmann, V.; Woelfle, U.; Rau, T.; Sauter G.; Heukeshoven, J.; Pantel, K. Changes in cytoskeletal protein composition indicative of an epithelial-mesenchymal transition in human micrometastatic and primary breast carcinoma cells. Clin Cancer Res **2005**, *11*, 8006-8014.

19. Woelfle, U.; Sauter, G.; Santjer, S.; Brakenhoff, R.; Pantel, K. Down-regulated expression of cytokeratin 18 promotes progression of human breast cancer. Clin Cancer Res **2004**, *10*, 2670-2674.