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ANA CECÍLIA DE ALBUQUERQUE OLIVEIRA

MARCADORES MOLECULARES NOS CÂNCERES DE MAMA E CERVICAL: ANÁLISES *IN SILICO* E *IN VITRO*

Recife
2018

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Tese apresentada ao Programa de Pós-Graduação em Biologia Aplicada à Saúde, da Universidade Federal de Pernambuco, como requisito parcial para obtenção do título de Doutor em Biologia Aplicada à Saúde.

Área de concentração: Biologia Aplicada à Saúde

Orientador: Prof. Dr. José Luiz de Lima Filho

Coorientador: Prof [a]. Dr [a]. Danyelly Bruneska Gondim Martins

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Aprovada em: Recife, 13 de junho de 2018:

Prof. Dr. José Luiz de Lima Filho
Orientador
Departamento de Bioquímica, LIKA - UFPE

Prof. Dr. Fabrício Oliveira Souto
Núcleo de Ciências da Vida - NCV, Centro Acadêmico do Agreste - CAA, LIKA, UFPE

Prof. Dr. Rafael Lima Guimarães
Departamento de Genética, LIKA, UFPE

Prof. Dr. Gustavo Alves do Nascimento
Núcleo de Ciências da Vida - NCV, Centro Acadêmico do Agreste - CAA, LIKA, UFPE

Prof[a]. Dr[a]. Rosângela Ferreira Frade de Araújo
Departamento de Bioquímica, LIKA, UFPE

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"Se eu enxerguei mais distante, foi por estar sobre ombros de gigantes."
Isaac Newton

RESUMO

Introdução: O câncer de mama é uma doença de grande impacto em mulheres no Brasil e no mundo. O câncer cervical, que está normalmente associado à infecção pelo vírus HPV, é a quarta causa de morte por câncer no país. Devido ao alto impacto destes dois tipos de cânceres na vida das mulheres em particular, identificar moléculas biomarcadoras que auxiliem tanto no diagnóstico, quanto no tratamento torna-se missão de relevância no campo científico. Estas moléculas biomarcadoras podem ser do tipo genético, epigenético, proteômico e podem ser usadas para o diagnóstico do câncer e seguimento da doença. Neste contexto o DEK, eNOS e TCF7L2 têm se mostrado bons candidatos a marcadores em cânceres como o de mama e o cervical. Objetivos: Identificar e avaliar moléculas de interesse em câncer de mama; estudar as interações do gene *DEK* com moléculas relevantes no câncer de mama e cervical; avaliar os níveis de expressão dos genes *NOS3* e *TCF7L2* em amostras de pacientes com câncer de mama; avaliar a presença de polimorfismos nos genes *NOS3* e *TCF7L2* em amostras de pacientes com câncer de mama. Metodologia: Foi realizado um estudo *in silico* para avaliar as possibilidades de interação dessas moléculas no contexto da célula com câncer de mama. Foi usado o banco de dados Pubmed para a metanálise. Foram realizadas análise de expressão gênica assim como analisados 3 polimorfismos do NOS3 e 3 polimorfismos do TCF7L2. Resultados: Na metanálise, foram encontrados 89 artigos, dos quais 20 foram selecionados para investigar a associação entre a eNOS e o câncer de mama. Foi observada a utilização desta proteína como biomarcador para câncer de mama. Nos testes experimentais, 22 amostras de pacientes com câncer foram analisadas e observou-se uma relação significativa na associação do *NOS3* c.-813C>T para histórico familiar de câncer (p=0.022). Conclusão: O gene DEK apresenta uma correlação clara com o desenvolvimento dos cânceres em mulheres, sendo ele, entre os marcadores estudados, o melhor candidato tanto para uma associação a alvos terapêuticos como para marcador de prognóstico nos cânceres de mama e cervical. Apesar do TCF7L2 contribuir em vias que podem levar à super expressão de proliferação em células mamárias, não houve significância estatística nos resultados encontrados. Verificou-se que a eNOS tem potencial como biomarcador para câncer de mama.

Palavras-chave: câncer de mama. câncer cervical. HPV. eNOS. TCF7L2. DEK.

ABSTRACT

Introduction: Breast cancer has a hugh impact in women in Brazil and all over the word. Cervical cancer is associated with HPV infections and figures at fourth cause of death in the country. On account of the high impact of these cancers in women life, to identify biomarkers that may assist in both the prognostic and treatment become a relevant mission in cientific field. These biomarkers can be genetic, epigenetic, proteomic and can be used in cancer diagnostic and in follow up of disease. In this context DEK, eNOS and TCF7L2 has been shown as promising candidats for breast and cervical cancer biomarkers. Objectives: To identify and evaluate relevant molecules for breast and cervical cancer; study the DEK gene interections with relevant molecules in breast and cervical cancer; evaluate the expression levels of *NOS3* and *TCF7L2* genes in samples of breast cancer patients; evaluate the presence of polymorphism in genes of *NOS3* and *TCF7L2* in samples of breast cancer patients. Metodology: an *in silico* study was carried to evaluate the possibilities of interection of these molecules in breast cancer cell context. The Pubmed databank was used to the meta-analysis. Gene expression analysis was performed as well as the analysis of 3 polymorphisms of NOS3 and 3 polymorphisms of TCF7L2. Results: In the meta-analysis, 89 articles were found, of which 20 were selected to investigate the association between eNOS and breast cancer. Use of this protein as a biomarker for breast cancer has been observed. In the experimental tests, 22 samples from cancer patients were analyzed and a significant association was observed between NOS3 c.-813C> T and family history of cancer ($p = 0.022$). Conclusion: The DEK gene has a clear correlation with the development of cancers in women, being the best candidate for both therapeutic targets and prognostic marker in breast and cervical cancers. Although TCF7L2 contributes to pathways that may lead to overexpression of mammary cell proliferation, there was no statistical significance in the results found. It has been found that eNOS has potential as a biomarker for breast cancer.

Keywords: breast cancer. cervical cancer. HPV. eNOS. TCF7L2. DEK.

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LISTA DE ABREVIATURAS E SIGLAS

ADP	Adenosina Difosfato (do inglês: *Adenosine diphosphate*)
AKT	PKB – Proteína quinase B (do inglês: *Protein kinase B*)
AML	Leucemia mieloide aguda (do inglês: *Acute myeloid leukemia*)
APC	Polipose adenomatosa coli (do inglês: *Adenomatosis polyposis coli*)
ATM	Ataxia Telangiectasia Mutado (do inglês: *Ataxia telangiectasia mutated*)
BH4	Tetrahidrobiopterina (do inglês: *Tetrahydrobiopterin*)
BRCA	Câncer de mama (do inglês: *Breast cancer*)
BRCA1	Proteína de susceptibilidade ao câncer de mama, tipo 1 (do inglês: *Breast cancer type 1*)
BRCA2	Proteína de susceptibilidade ao câncer de mama, tipo 2 (do inglês: *Breast cancer type 2*)
BRIP1	Proteína de interação BRCA1 (do inglês: *BRCA1 interacting protein*)
Ca^{2+}	Íon de cálcio
CAV1	Caveolina 1 (do inglês: *Caveolin 1*)
CCL2	Quimiocina (C-C motif) Ligante 2 (do inglês: *Chemokine (C-C motif) ligand 2*)
CHEK2	Quinase 2 de ponto de verificação (do inglês: *Checkpoint kinase 2*)
CpG	Ponte citosina-fosfato-guanina (do inglês: *Cytosine-phosphate-guanine*)
DNA	Ácido desoxirribonucleico (do inglês: *Deoxyribonucleic acid*)
E2F	Proteína associada ao retinoblastoma (do inglês: *Retinoblastoma protein*)
EGFR	Receptor do fator de crescimento epidermal (do inglês: *Epidermal growth factor receptor*)
EMT	Transição epitelial-mesenquimal (do inglês: *Epithelial–mesenchymal transition*)
eNOS	Óxido nítrico sintase endotelial (do inglês: *Endothelial nitric oxide synthases*)
ER	Receptor de estrógeno (do inglês: *Estrogen receptor*)

FAD	Flavina adenina dinucleotídeo (do inglês: *Flavin-adenin-dinukleotid*)
FMN	Flavina mononucleotídeo (do inglês: *Flavin mononucleotide*)
HER2	Fator de crescimento epidérmico humano 2 (do inglês: *Human epidermal growth factor receptor 2*)
HIF-1α	Fator indutor da hipóxia 1α (do inglês: *Hypoxia-inducible factor 1-α*)
HPV	Papiloma vírus humano (do inglês: *Human papiloma virus*)
IGF-1R	Receptor do fator de crescimento 1 tipo insulina (do inglês: *Insulin-like growth factor receptor-1*)
IHC/FISH	Imunohistoquímica/Hibridização por fluorescência *in situ* (do inglês: *Immunohistochemistry/Fluorescence* in situ *hybridization and immunohistochemistry*)
INCA	Instituto Nacional do Câncer
iNOS	Óxido nítrico sintase induzida (do inglês: *Inducible nitric oxide synthase*)
MCP-1	Proteína 1 quimioatrativa de monócitos (do inglês: *Monocyte chemoattractant protein-1*)
MS	Ministério da Saúde
mTOR	Alvo da rapamicina mamífera (do inglês: *Mammalian target of rapamycin*)
NADPH	Nicotinamida adenina dinucleotídeo fosfato (do inglês: *Nicotinamide adenine dinucleotide phosphate*)
nNOS	Óxido nítrico sintase neuronal (do inglês: *Neuronal nitric oxide synthase*)
NO	Óxido nítrico (do inglês: *Nitric oxide*)
NOS	Óxido nítrico sintase (do inglês: *Nitric oxide synthase*)
OPN	Osteopontina (do inglês: *Osteopontin*)
p53	Proteína tumoral 53 (do inglês: *Tumor protein p53*)
P70-S6	Proteína ribosomal S6 quinase β-1 (S6K1) (do inglês: *Ribosomal protein S6 kinase β-1*)
PAM50	Ensaio de prognóstico de assinatura de gene de câncer de mama Prosigna (do inglês: *Prosigna Breast Cancer Prognostic Gene Signature Assay*)

PCK	Proteína quinase C (do inglês: *Protein kinase c*)
PCR	Reação em cadeia da polimerase (do inglês: *Polymerase chain reaction*)
PGE2	Prostaglandina 2 (do inglês: *Prostaglandin E2*)
PI3K	Fosfatidilinositol-4,5-bisfosfato 3-quinase (do inglês: *Phosphatidylinositol-4,5-bisphosphate 3-kinase*)
PIP2	Fosfatidilinositol 4,5-bifosfato (do inglês: *Phosphatidylinositol 4,5-bisphosphate*)
PIP3	Fosfatidilinositol (3,4,5)-trifosfato (do inglês: *Phosphatidylinositol (3,4,5)-trisphosphate*)
PR	Receptor de progesterona (do inglês: *Progesterone receptor*)
pRb	Proteína Retinoblastoma (do inglês: *Retinoblastoma protein*)
PTEN	Homólogo da tensina e fosfatase (do inglês: *Phosphatase and tensin homolog*)
RAD51C	Proteína membro da família RAD51
RAD6B	UEB2B – Enzima Conjugadora de Ubiquitina E2 B (do inglês: *UEB2B – Ubiquitin Conjugating Enzyme E2 B*)
RNA	Ácido Ribonucléico (do inglês: *Ribonucleic acid*)
RNS	Espécies reativas de nitrogênio (do inglês: *Reactive nitrogen species*)
ROS	Espécies reativas de oxigênio (do inglês: *Reactive oxygen species*)
RPA-1	Proteína repressora transcricional-1 (do inglês: *Repressor activator protein 1*)
SFRP	Proteína secretada relacionada ao frisado (do inglês: *Secreted frizzled-related protein*)
SNP	Polimorfismo de nuceotídeo único (do inglês: *Single nucleotide polymorphism*)
TCF	Fator de transcrição (do inglês: *Transcription factor*)
TCF7L2	Fator de Transcrição 7 tipo 2 (do inglês: *TCF7L2 transcription factor 7 like 2*)
TNBC	Câncer de mama triplo negativo (do inglês: *Triple negative breast cancer*)

VEGF	Fator de crescimento vascular endotelial (do inglês: *Vascular endothelial growth factor*)
VNTR	Número variado de repetição em tandem (do inglês: *Variable number tandem repeats*)
Wnt	Via de sinalização

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1 INTRODUÇÃO

A saúde da mulher tem sido tratada, progressivamente, com mais zelo por parte do poder público no Brasil há alguns anos. Em 1983 e em 2004 foram fincados marcos importantes para o avanço neste tema com o lançamento, respectivamente, do programa *Assistência Integral à Saúde da Mulher: bases de ação programática* e da *Política Nacional de Atenção Integral à Saúde da Mulher - Princípios e Diretrizes*. Estas iniciativas visam promover a melhoria das condições de vida e da saúde das brasileiras por meio de ações que ampliam o acesso aos serviços de promoção, prevenção, assistência e recuperação da saúde. Os esforços têm se concentrado no desenvolvimento de ações em temas estratégicos, como: planejamento reprodutivo (métodos contraceptivos), atenção obstétrica (pré-natal, parto, puerpério, urgências e emergências obstétricas e aborto), vigilância epidemiológica do óbito materno, violência sexual e doméstica, climatério, saúde mental, feminilização da AIDS, infecções sexualmente transmissíveis e câncer de colo de útero e mama (MS, 2017).

Dentre os tipos do câncer nas mulheres, o de mama é o mais prevalente, com grande incidência tanto em países desenvolvidos (794.000 casos) como em países em desenvolvimento ou subdesenvolvidos (883.000 casos), segundo o último relatório global de câncer (GLOBOCAN, 2012). Considerando os dois sexos, o câncer de mama é o segundo mais frequente no mundo, com 1,68 milhão de novos casos em 2012. Já o câncer cervical, neste mesmo estudo, foi o quarto mais comum em mulheres e o sétimo entre homens e mulheres, com 528.000 casos estimados no mundo (FERLAY et al., 2014).

Existem vários fatores que influenciam no desenvolvimento do câncer, tais como: infecção por vírus, obesidade e o aspecto comportamental, como tabagismo e alcoolismo (FENGA, 2016). No câncer de mama, a circunstância que está associada à maior predisposição para o desenvolvimento da doença é o histórico familiar positivo. Pacientes de ambos os gêneros com casos de familiares de primeiro grau com câncer têm o risco aumentado em duas vezes, podendo atingir até cinco vezes, mediante o número de membros afetados na família (MISHRA; VERMA, 2010). Fatos como estes apontam a relevância do componente hereditário no risco da ocorrência do câncer de mama, assim como a presença de mutações também pode ser um fator determinante no desenvolvimento do câncer (PÉREZ-SOLIS et al., 2016).

O teste citológico ainda é a forma mais utilizada para detecção de lesões pré-cancerosas e câncer cervical. No entanto, sua sensibilidade é de apenas 60 a 70% na detecção da presença da lesão (CONG; COX; CANTOR, 2007). Biomarcadores específicos para o HPV como p16ink4a, HPV E6/E7 mRNA, ou marcadores de metilação, servem como marcadores secundários, mediante o teste positivo para a presença do DNA viral (INCA, 2018).

Um biomarcador é uma molécula que, quando detectada, pode indicar a existência da doença a ele associada. Em casos de câncer, a constatação da sua presença permite

ajustar o direcionamento terapêutico ou mesmo avaliar a sobrevida do paciente. Estas moléculas podem ser secretadas pelo tumor ou ser provenientes de uma resposta específica à presença das células tumorais. Marcadores genéticos, epigenéticos, proteômicos, glicômicos e marcadores por imagem podem ser usados tanto para o diagnóstico quanto para o acompanhamento do câncer. Contudo, enquanto alguns marcadores apresentam uma alta sensibilidade e especificidade para a detecção do câncer, outros ainda não se encontram prontos para o uso humano, pois precisam ser validados na detecção de doenças, diagnóstico e monitoramento da evolução de quadros clínicos (WALSH et al., 2016).

Neste contexto, a presente tese teve os objetivos apontados a seguir.

1.1 OBJETIVOS

- Geral
  - Identificar e avaliar moléculas relacionadas ao desenvolvimento de carcinogênese em mulheres, efetuando análises *in silico* e *in vitro* ao nível de expressão gênica e presença de polimorfismos.
- Específicos
  - Estudar as interações do gene *DEK* com moléculas relevantes nos cânceres de mama e cervical.
  - Avaliar os níveis de expressão dos genes *NOS3* e *TCF7L2* em amostras de pacientes com câncer de mama.
  - Avaliar a presença de polimorfismos nos genes *NOS3* e *TCF7L2* em amostras de pacientes com câncer de mama.

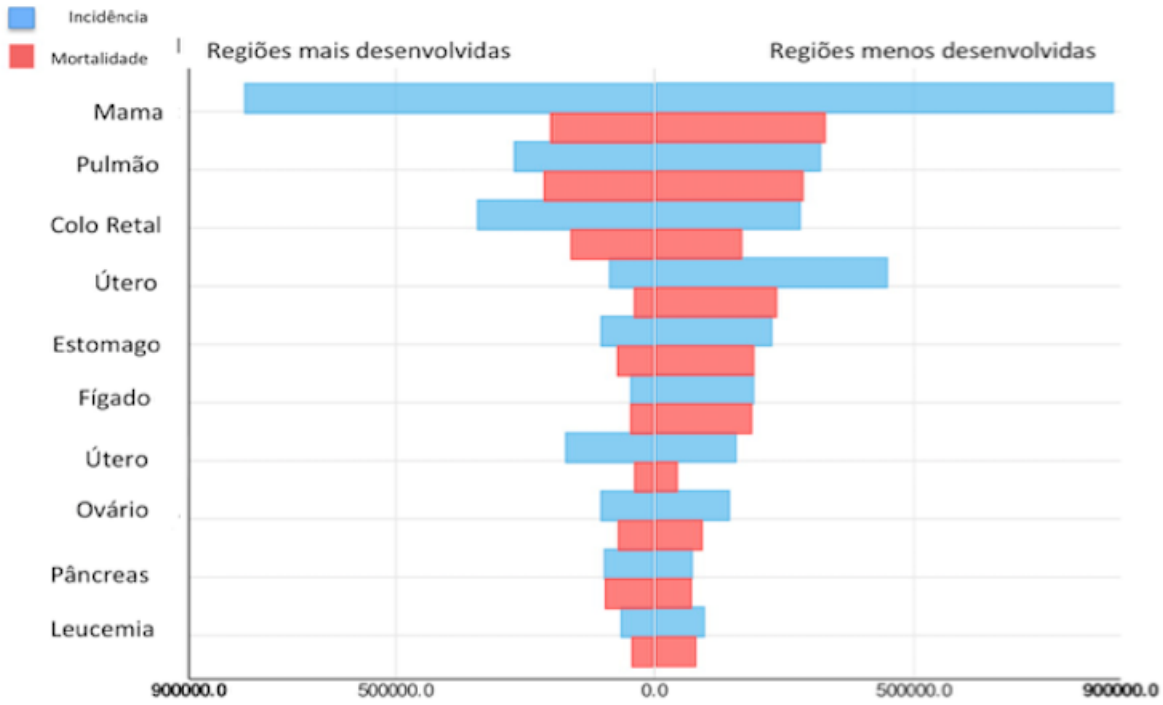
2 REVISÃO DA LITERATURA

2.1 ESTATÍSTICAS DO CÂNCER

Segundo o Globocan, no ano de 2012, houve uma incidência de 14,1 milhões de novos casos de câncer (FERLAY et al., 2014). O câncer de mama é o mais frequente entre mulheres no mundo, tendo uma grande incidência independente do grau de desenvolvimento do país (Figura 1). Para o Brasil, estimam-se 59.700 casos novos de câncer de mama, para cada ano do biênio 2018-2019 (CATTEAU et al., 1999). Em regiões menos desenvolvidas, o câncer de mama atinge cerca de 324.000 mulheres, seguido pelo câncer de pulmão e cérvix (281.000 e 230.000), respectivamente. O câncer de mama é o quinto mais mortal no mundo: 522.000 óbitos em 2012 (FERLAY et al., 2014).

O câncer de mama também pode acometer homens, mas em pequenas proporções, representando cerca de 1% do total de casos da doença (ANDERSON et al., 2009). É raro que o câncer de mama se manifeste antes dos 35 anos, porém a cima desta idade sua incidência cresce progressivamente, especialmente após os 50 anos de idade (RIZZOLO et al., 2011).

Figura 1 – Número estimado de mortalidade e incidência dos 10 tipos de câncer mais frequentes em mulheres no ano de 2012.



Fonte – Adaptado de GLOBOCAN (2012).

Já o câncer cervical vitimou 266.000 mulheres em todo o mundo no ano de 2012, o que corresponde a 7,5% de todas as causas de morte de mulheres. A taxa de incidência e mortalidade deste tipo de câncer apresenta uma variação bastante elevada em relação ao grau de desenvolvimento do país. Enquanto que os dados apontam para altas incidência (444.500 casos) e mortalidade (230.200 mortos) em países pouco desenvolvidos como os da África central e Oriental, as regiões desenvolvidas como América do Norte, Austrália e Europa apresentam incidência (83.100 casos) e mortalidade (35.500) por câncer cervical mais baixas. O risco de morte por câncer cervical antes dos 75 anos é três vezes maior em regiões pouco desenvolvidas em relação às regiões desenvolvidas (FERLAY et al., 2014).

No Brasil, segundo o INCA, o câncer cervical é a quarta causa de morte de mulheres por câncer, com 16.340 novos casos em 2016 e estimativa de 16.370 em 2018. Apesar do crescimento nas estimativas de incidência do câncer cervical, o país avançou na sua capacidade de realizar diagnóstico precoce, pois na década de 1990 70% dos casos diagnosticados eram da forma invasiva. Atualmente 44% dos casos são de lesão precursora do câncer, chamada *in situ*, ou localizada (CATTEAU et al., 1999).

Em regiões menos desenvolvidas, agentes infecciosos, como o HPV, são uma das maiores causas de câncer (MARTEL et al., 2012). A introdução de programas de vacinação e tratamento nos programas de saúde, são de grande impacto em um futuro controle do câncer. (FERLAY et al., 2014)

2.2 CÂNCER DE MAMA

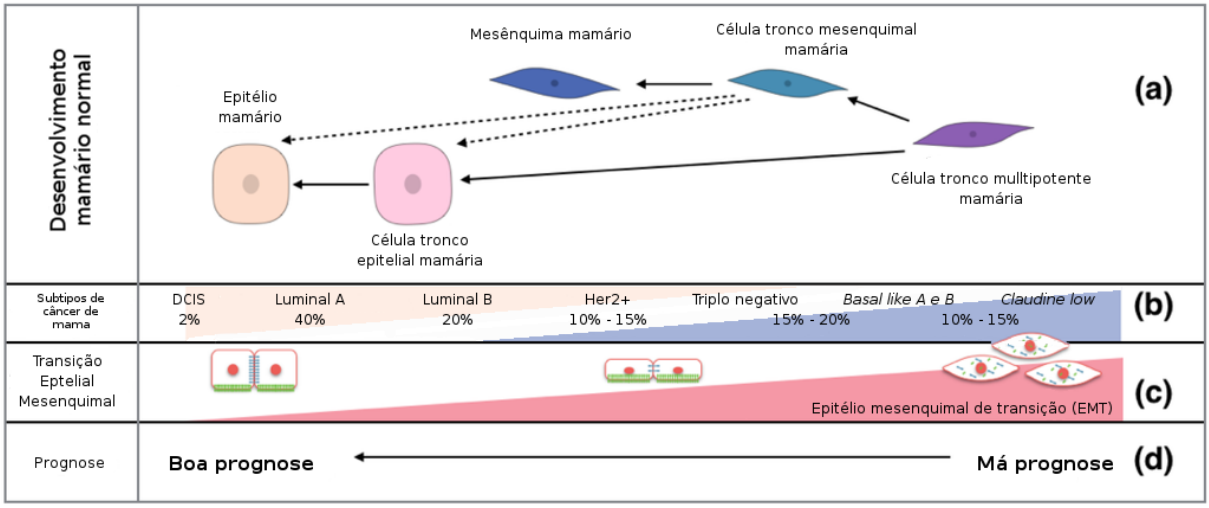
Do ponto de vista clínico, o câncer de mama pode ser classificado a partir do perfil de imunohistoquímica e hibridização *in situ* (IHC/FISH) e dividido com base na presença de receptores de estrógeno (ER), receptores de progesterona (PR) ou receptor do fator de crescimento epidermal humano 2 (HER2) (Figura 2). No nível molecular, Perou et al. (2000) analisaram o padrão da expressão de genes de câncer de mama, derivados de microarrays de cDNA, identificando inicialmente 4 assinaturas mais representativas: luminais, HER-2, *basal-like* e o subtipo *normal breast-like subtype*. Estudos subsequentes levaram à divisão de tumores luminais em dois grupos (A e B) que demonstraram uma correlação entre os padrões de expressão gênica, sobrevivência, reincidência, local de metástases e resposta à quimioterapia (SORLIE et al., 2001; ROUZIER et al., 2005).

Após alguns anos, outros subtipos moleculares foram descritos, como *Claudin Low* e tumores apócrinos (PARKER et al., 2009) capaz de reanalizar os 5 grupos, definindo então os 4 subtipos mais importantes, atualmente conhecidos: Lumina A, Luminal B, HER-2 e *Basal Like*.

Subsequentemente, 5 novos testes de prognóstico foram desenvolvidos para o câncer de mama: MamaPrint, MapQuant Dx, Oncotype DX, PAM50 e o Theros Breast Cancer

Index. A lógica envolvida no desenvolvimento de testes baseados em vários genes seria não só a obtenção de dados preditivos e de prognóstico, mas que estes dados sejam mais confiáveis do que apenas os dos ensaios baseados em imunohistoquímica, reduzindo erros técnicos e interpretações subjetivas (PRAT; ELLIS; PEROU, 2011; TOSS; CRISTOFANILLI, 2015).

Figura 2 – As células tronco mamárias e o tipo de câncer correspondente. (a) Expansão de células mamárias a partir de uma progenitora, (b) Classificação subcelular de cânceres de mama, de acordo com suas origens e porcentagem de prevalência clínica; (c) Envolvimento de EMT de acordo com a subclassificação do câncer de mama ao nível sugerido de EMT; (d) A prognose associada a subtipos de câncer de mama.



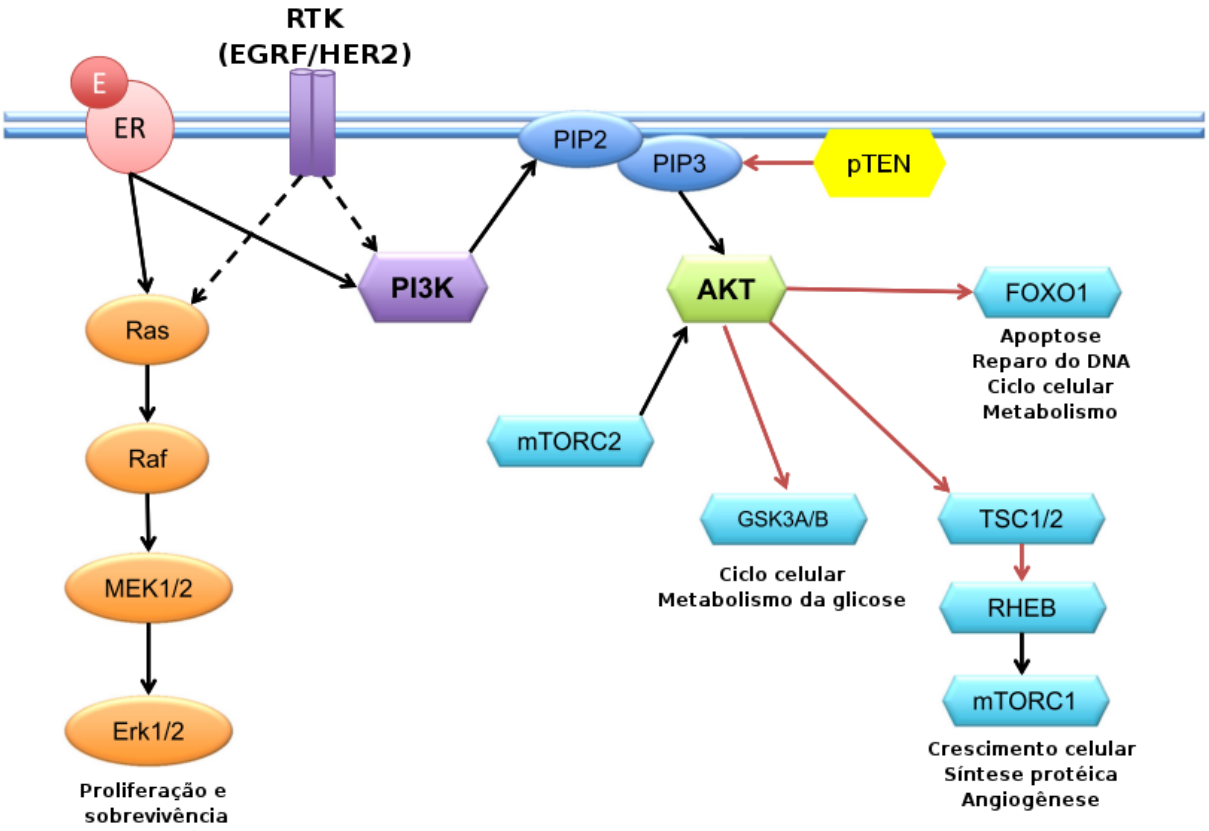
Fonte – Adaptado de Flemban e Qualtrough (2015).

A via PI3K/AKT/mTOR e sua comunicação com as vias RAS/RAF/MEK/MAPK tem um papel crucial no crescimento, sobrevivência, diferenciação e proliferação da célula neoplásica (Figura 3). Além disso, a via PI3K/AKT/mTOR participa no controle do complexo de energia celular, metabolismo da glicose, senescência e angiogênese, e em células doentes ER positivas, atua promovendo atividade transcricional, que é ativada pela presença de estrógeno (TOSS; CRISTOFANILLI, 2015).

Fosfoinosotol 3-quinase (PI3K) é uma proteína do tipo quinase, recrutada para a membrana celular pelos receptores de fatores de crescimento ativados, incluindo HER2, receptor do fator de crescimento epidermal (EGFR) e fator de crescimento do tipo insulina 1 (IGF-1R). O PI3K fosforila o grupo *phosphatidylinositol (4,5)-bisphosphate* (PIP2) para produzir *phoshatidylinositol-3,4,5-trisphosphate* (PIP3). Este por sua vez é o segundo mensageiro que sinaliza, através da via AKT, a ativação de algumas enzimas quinases e fatores de transcrição, incluindo a *mammalian target of rapamycin* (mTOR). A via RAS/RAF/MEK/MAPK converge com a PI3K/AKT e é reconhecida como um ativador alternativo da mTOR. Por outro lado, o homólogo da fosfatase e tensina (PTEN) catalisa a desfosforilação de PIP3, atuando como um inibidor da atividade do PI3K. Em paralelo

com a ativação dos receptores dos fatores de crescimento, os estrógenos podem ativar o ER nuclear, pela via genômica, ou o ER da membrana, pela via não genômica. ERs associados à membrana ligam-se ao PI3K ativando moléculas como AKT e RAS, que vão dar sequência a eventos de proliferação, crescimento celular e angiogênese. As proteínas quinases envolvidas nessas vias representam alvos atrativos no tratamento do câncer de mama. Já há algumas moléculas em desenvolvimento nas fases pré-clínica e clínica (TOSS; CRISTOFANILLI, 2015).

Figura 3 – Via PI3K/AKT/mTOR e via RAS/RAF/MEK/MAPK envolvidas na proliferação celular, crescimento, síntese proteica e angiogênese



Fonte – Adaptado de Toss e Cristofanilli (2015).

Os biomarcadores são fundamentais para auxiliar os médicos no diagnóstico, predição e tratamento de câncer de mama, além do estadiamento, predição da resposta ao tratamento e na melhora da taxa de sobrevivência das pacientes. Mais e melhores informações permitem uma prescrição da terapia melhor direcionada. No caso do câncer de mama primário, o desenvolvimento de técnicas moleculares de identificação permitiram prognósticos mais precisos, além de trazer informações preditivas aos biomarcadores convencionais como ER, PR e EGFR (EIFEL et al., 2001).

A quimioterapia, seguida por cirurgia ou por radioterapia, e a terapia adjuvante são as principais formas de tratamento e, na maioria dos casos, conseguem mais de 50%

de redução tumoral (TIMP; FEINBERG, 2013). Graças a exames como a mamografia, os índices de mortalidade têm decrescido entre os pacientes, como resultado de um diagnóstico precoce (VERMA; MANNE, 2006). Como não é possível prever o risco de metástases, a maioria dos pacientes recebem a quimioterapia adjuvante, o que ajuda a erradicar células doentes que já se espalharam por locais distantes antes mesmo do diagnóstico. Apesar de melhorar os índices de sobrevivência, a terapia adjuvante apresenta uma série de efeitos secundários que afetam a qualidade de vida destes pacientes, como visto na Tabela 1. Na realidade, mulheres que podem ser curadas através de um tratamento local, incluindo cirurgia e radioterapia, para que a reincidência do tumor seja evitada, são submetidas a medidas profiláticas com efeitos tóxicos (LAU et al., 2009).

Tabela 1 – Cardiotoxicidade dos agentes terapêuticos.

Agente	Potencial cardiotoxicidade
Antraciclinas: Doxorrubicina e Epirrubicina	Falha cardíada congestiva, cardiomiopatia
Agentes alquilantes	Falha cardíada congestiva
Taxanos: paclitaxel e docetaxel	Falha cardíada congestiva, iquemia, arritmia
Terapias alvos: Trastuzumab, lapatinib	Falha cardíada congestiva
Bloqueio hormonal: Tamoxifeno	Tromboembolias

Fonte – Adaptado de Bodai e Tuso (2015).

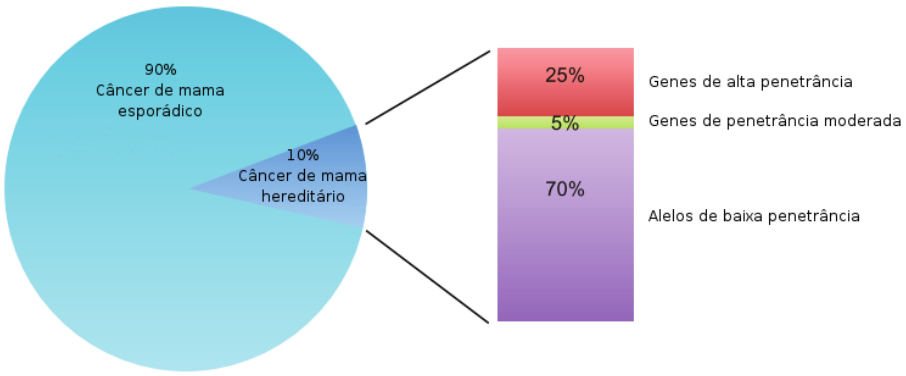
2.3 GENÉTICA E EPIGENÉTICA DO CÂNCER

Cerca de 10% de todos os cânceres de mama são hereditários, causados por mutações herdadas em células germinativas, enquanto que em 90% dos demais casos o câncer de mama está relacionado às mutações somáticas e alterações epigenéticas (Figura 4). Genes de alta penetrância (BRCA1 and BRCA2) contribuem com 25% do fator hereditário. Genes de penetrância moderada (CHEK2, ATM, PALB2, BRIP1, RAD51C) contribuem com menos de 5% do risco em desenvolver o câncer de mama (RIZZOLO et al., 2011).

Uma série de alterações somáticas, incluindo mutações e amplificações gênicas, estão relacionadas à etiologia no câncer de mama. A hipermetilação da região promotora de genes envolvidos no reparo do DNA e de sinalização celular mediada por hormônios, assim como a expressão alterada de micro RNAs direcionados à regulação de genes importantes no controle do câncer de mama, atuam de forma equiparada ao papel de fatores genéticos no desenvolvimento do câncer. A elucidação das alterações genéticas e epigenéticas envolvidas no câncer de mama, tanto herdadas quanto adquiridas, podem desvendar vias moleculares envolvidas no desenvolvimento e progressão do câncer de mama, além de ter um impacto clínico e terapêutico importante na melhoria dos cuidados prestados aos pacientes doentes (RIZZOLO et al., 2011).

Epigenética, segundo Weinhold (2006), significa literalmente “além de mudanças na sequência genética”, anotando que “O termo evoluiu para incluir qualquer processo

Figura 4 – Susceptibilidade genética no câncer de mama hereditário.



Fonte – Adaptado de Rizzolo et al. (2011).

que altere a atividade do gene, sem alterar a sequência do DNA, e leva a modificações que podem ser transmitidas para as células dessa provenientes (embora as experiências mostrem que algumas mudanças epigenéticas podem ser revertidas)". A epigenética está relacionada às interações entre genes e o ambiente celular. Essas interações conferem características únicas de cada pessoa e permitem um controle dinâmico da expressão de vários genes críticos à célula. Alterações epigenéticas podem levar ao desenvolvimento de doenças como o câncer. Desta forma, a epigenética do câncer representa um grande potencial farmacêutico contra as neoplasias (BHATTACHARJEE; SHENOY; BAIRY, 2016). Frequentemente a remodelagem das histonas pode resultar de metilações, ubiquitinação, fosforilação, biotinização sumoilação, ADP ribosilação e acetilação nos grupos N-terminais dos resíduos de lisina. No entanto, a acetilação e a metilação estão entre as modificações mais estudadas e são consideradas as mais prevalentes (KOUZARIDES, 2007; LI; DANIEL; TOLLEFSBOL, 2011; DANIEL; TOLLEFSBOL, 2015).

A acetilação do resíduo de lisina da histona remove sua carga positiva, o que reduz a atração ao DNA, negativamente carregado, produzindo uma perda da estrutura inicial da cromatina. Essa conformação aberta permite que vários fatores transcricionais acessem o DNA promovendo a ativação da transcrição dos genes (CLAYTON; HAZZALIN; MAHADEVAN, 2006).

A metilação do DNA é um dos eventos mais importantes envolvidos na iniciação e progressão do câncer. Este processo envolve a adição de grupos metil no carbono 5' nos resíduos de citosina, que precedem guaninas, ligados por grupos de pontes de fosfato (CpG), através de um doador de metil como a S-adenosilmetionina. Esta metilação é capaz de deter o processo de transcrição, inibindo a ligação de fatores transcricionais com os locais alvo (BIRD, 2002) As citosinas metiladas, por sua vez, atuam como locais para ancoragem de várias proteínas de ligação ao DNA metilado (MBD1, MBD2, MBD3, and Mecp2),

que são reconhecidas por enzimas modificadoras de histonas, tais como deacetilases de histonas (HDACs), que são capazes de promover a repressão gênica (CURRADI et al., 2002; LUCZAK; JAGODZIńSKI, 2006).

Os tumores humanos apresentam uma perda massiva da metilação do DNA, mas também adquirem padrões específicos de hipermetilação em alguns promotores (HERMAN; BAYLIN, 2003; FEINBERG; TYCKO, 2004). Além disso, essas mudanças estão relacionadas à presença de aberrações nos padrões de modificação das histonas (ESTELLER, 2007).

Os genes supressores tumorais codificam proteínas que controlam a capacidade de proliferação e sobrevivência de uma célula. Esses genes podem ser submetidos a múltiplos mecanismos que desregulam suas funções durante o processo oncogênico. Podemos incluir entre esses processos as mutações, deleções, rearranjos genéticos e silenciamento epigenético da transcrição. Alguns genes supressores tumorais, como o supressor tumoral 53 (p53), podem ser inativados primariamente por mutações (MAJEWSKI; BERNARDS, 2011; WEIGEL; DOWSETT, 2010). Apesar da formação do câncer estar diretamente relacionada às mutações, as modificações epigenéticas são características de todos tipos de câncer, desde as células precursoras às metástases mais avançadas. São essas modificações epigenéticas que garantem uma heterogeneidade ao tumor (WEIGEL; DOWSETT, 2010). A explicação mais simples dessas relações é que o câncer é causado pela desregulação epigenética, o que pode gerar um elevado grau de variações fenotípicas observadas entre cânceres individuais e que levam à seleção de células resistentes no tumor (WEIGEL; DOWSETT, 2010). Acredita-se que o silenciamento epigenético de genes supressores tumorais seja um dos primeiros eventos no processo oncogênico (MAJEWSKI; BERNARDS, 2011).

2.4 BIOMARCADORES DO CÂNCER

A heterogeneidade do câncer pode ser controlada através da identificação de moléculas confiáveis relacionadas à doença e seus biomarcadores. Estes biomarcadores são moléculas biológicas como DNA, proteínas, lipídios, metabolitos, ou propriedades biológicas como apoptose, proliferação, angiogenese, tensão de oxigênio ou ainda características clínicas que podem ser medidas e avaliadas de forma objetiva como indicadores de processos patológicos ou resposta terapêutica em pacientes com câncer (LUDWIG; WEINSTEIN, 2005; MISHRA; VERMA, 2010; RICHMOND; DUNN, 2012; MÄBERT et al., 2014).

Os marcadores moleculares tumorais são produzidos dentro do tumor ou acumulados em outros tecidos e fluidos do corpo humano em resposta à presença do câncer. Existem dois outros tipos de biomarcadores, os relacionados à doença e os relacionados às drogas do tratamento (MÄBERT et al., 2014). Apesar de todos os esforços, não existem muitos biomarcadores validados para o uso clínico. Avanços nas pesquisas podem ajudar a acelerar

a descoberta de moléculas adicionais e principalmente seu uso no diagnóstico (LU; ZHANG; ZHANG, 2017).

Entretanto, existem alguns desafios na descoberta de novos biomarcadores para o câncer, tais como a validação e a utilização. Um biomarcador ideal deve ser confiável, ter um bom custo-benefício, ser medido de forma não invasiva ou minimamente invasiva, com alta especificidade e sensibilidade. Os fatores mais relevantes que podem dificultar a introdução de um novo biomarcador na rotina clínica são uma baixa especificidade, uma baixa sensibilidade e consequentemente baixo poder preditivo e de prognóstico (DIAMANDIS, 2012).

A sensibilidade é caracterizada pela porcentagem de amostras positivas identificadas por um marcador como positivas de fato, enquanto que especificidade é dada pela porcentagem de amostras negativas identificadas como negativas de fato. Para que seja aceito clinicamente, espera-se que um biomarcador tenha sensibilidade e especificidade próximas a 100%. No entanto, nenhum biomarcador conhecido possuei esta habilidade (ISSAQ; WAYBRIGHT; VEENSTRA, 2011).

De acordo com Issaq, Waybright e Veenstra (2011), as maiores dificuldades na migração de um biomarcador da bancada para o uso clínico podem estar relacionada a: erros no desenvolvimento dos estudos; grupo reduzido de amostras analisadas; os indivíduos utilizados como de caso e controle não coincidirem com faixa etária, sexo e raça pretendidos para o seu uso; e erros na execução do estudo, incluindo falta de padronização na coleta das amostras, manuseio e armazenagem do material, preparação e análise.

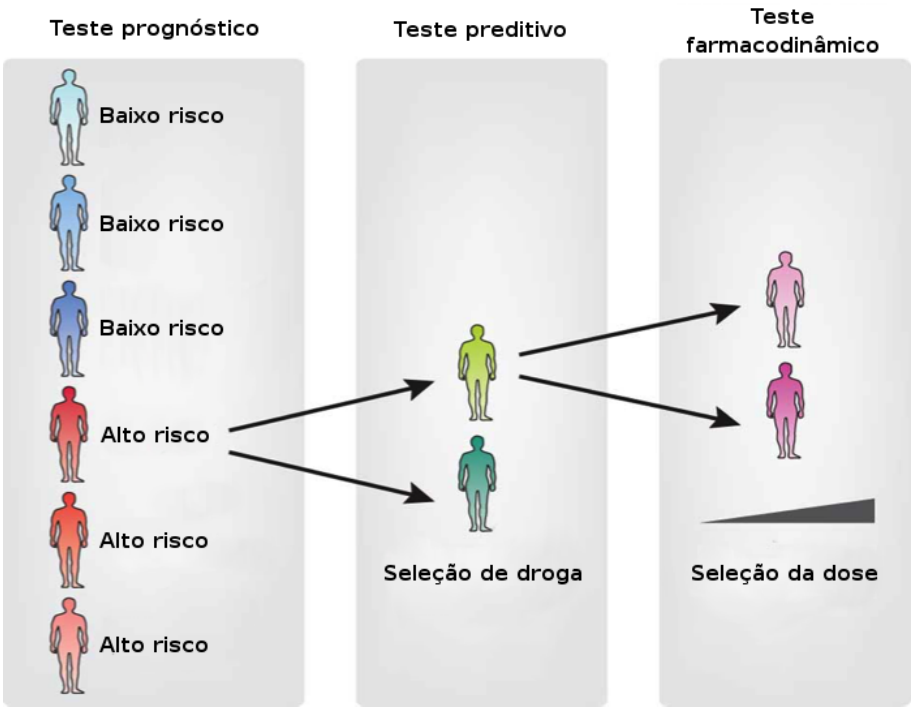
A ativação de vias de sinalização no câncer são frequentemente o resultado de alterações genômicas, como mutações, translocações e ganhos ou perdas em números de cópias, em componentes chave dessas vias. Tecnologias capazes de detectar essas modificações são ferramentas valiosas na identificação de biomarcadores para terapias direcionadas (MAJEWSKI; BERNARDS, 2011).

Os biomarcadores podem ser de grande ajuda em três decisões importantes que o médico precisa tomar durante o tratamento do paciente doente (Figura 5).

A primeira pergunta seria se o paciente necessita de uma terapia adicional (adjuvante) após a cirurgia do tumor primário. Para auxiliar nesta resposta, existe um marcador que é chamado de marcador de prognóstico, pois indica a possibilidade do tumor voltar na ausência de tratamento posterior. O grau histológico do tumor, que pode ser observado na análise patológica, já é um marcador de prognóstico convencional. E caso este marcador indique uma doença agressiva, um tratamento posterior é necessário para reduzir as chances de recorrência. Neste caso, o médico precisa considerar a terapia adequada. Em segundo lugar, está a predição. Marcadores que ajudam a predizer respostas a regimes terapêuticos específicos são chamados de marcadores preditivos. Uma das metodologias mais utilizadas

é a imunohistoquimica, que resulta em um biomarcador para detectar a presença aumentada do receptor de estrógeno no câncer de mama, já que apenas o câncer positivo para os receptores de estrógeno são capazes de responder à terapia hormonal. Uma terceira consideração a ser feita é a quantidade da droga que deve ser administrada para cada paciente individualmente. Um biomarcador que auxilia na seleção da dose ideal de uma determinada droga para cada paciente é conhecido como biomarcador farmacodinâmino (MAJEWSKI; BERNARDS, 2011).

Figura 5 – Testes utilizados no manejo de pacientes com câncer.



Fonte – Adaptado de Majewski e Bernards (2011).

O desenvolvimento de novos biomarcadores é fundamental para detecção e diagnóstico, determinação do risco, subtipagem da doença, predição da resposta ao tratamento e da sobrevivência, permitindo um tratamento personalizado do câncer. A integração entre novos biomarcadores e características já testadas como os tumores HR ou HER2 podem ajudar a direcionar as decisões terapêuticas tanto em tumores primários como nos metastáticos (TOSS; CRISTOFANILLI, 2015).

2.5 ÓXIDO NÍTRICO SINTASE ENDOTELIAL

O óxido nítrico ou *nitric oxide* (NO), a menor molécula sinalizadora conhecida, é produzido por três isoformas da NO sintase (NOS; EC 1.14.13.39). Todas elas utilizam a L-arginina e o oxigênio molecular como substratos e requerem os cofatores fosfato de dinucleótido de nicotinamida e adenina (NADPH), flavina adenina dinucleotídeo (FAD), flavina

mononucleotídeo (FMN) e (6R-)5,6,7,8-tetrahydrobiopterina (BH4) (FÖRSTERMANN; SESSA, 2012). O NO é uma molécula com múltiplas funções, pois apresenta vários alvos moleculares e controla funções regulatórias, tais como a de neurotransmissão (O'DELL et al., 1991) ou controle do tônus vascular (RAPOPORT; DRAZNIN; MURAD, 1983). O estímulo da NOS na ativação da guanilil ciclase, regula a transcrição gênica (PANTOPOULOS; HENTZE, 1995; LIU; HILL; HAILE, 2002) e pode causar modificações pós transcricionais de proteínas (ribosilação de adenosina di-fosfato (ADP)) (POZDNYAKOV et al., 1993; BRÜNE et al., 1994). Esse composto é capaz de gerar danos oxidativos e de causar s-nitrosilação de moléculas como proteínas, lipídios e do DNA (MIKKELSEN; WARDMAN, ; LEE; YANG; PARK, 2003).

Em mamíferos, as três formas da enzima NOS que levam à síntese de NO são conhecidas como neuronal (*nNOS* ou NOSI), a induzida (iNOS ou NOSII) e endotelial (eNOS ou NOSIII) (FÖRSTERMANN; SESSA, 2012). A nNOS está associada a uma variedade de eventos de sinalização nervosa, funções fisiológicas, além de estar relacionada ao aprendizado, memória e neurogênese (ZHOU; ZHU, 2009). A iNOS não é comumente expressa nas células, porém sua expressão pode ser desencadeada por estímulos celulares tais como citocinas, pela presença de patógenos e outros agentes (FÖRSTERMANN; SESSA, 2012). A expressão da enzima pode ser estimulada em qualquer célula ou tecido desde que os agentes de indução apropriado sejam identificados (FÖRSTERMANN et al., 1994; FÖRSTERMANN, 2000). Uma vez expressa, a iNOS é constantemente ativada e não sofre a regulação do Ca^{2+} intracelular (FÖRSTERMANN; SESSA, 2012).

A eNOS é a mais importante para a formação do NO no sistema cardiovascular (LACCHINI; SILVA; TANUS-SANTOS, 2010), com uma maior visibilidade por estar relacionada a problemas coronários e, mais recentemente, tumores mamários (FÖRSTERMANN et al., 1994; PERVIN; SINGH; CHAUDHURI, 2008). A eNOS é produzida por células endoteliais e a sua expressão aumentada é observada na vascularização de vários tecidos tumorais incluindo câncer de bexiga e cólon (KLOTZ et al., 1999; CHHATWAL et al., 1994). Trata-se de um mediador central dentre vários estimuladores de crescimento endoteliais, como o fator de crescimento endotelial vascular (VEGF) e a prostaglandina E2 (PGE2) (GAO et al., 2015).

Os benefícios ou riscos oferecidos pelo NO dependem da concentração, duração e níveis da exposição do tecido às espécies reativas de nitrogênio e oxigênio (RNS/ROS), somados à conformação genética do tecido e microambiente ao redor do tumor (THOMAS et al., 2004; PERVIN; SINGH; CHAUDHURI, 2001; PAQUETTE et al., 2001; FOLKES; WARDMAN, 2004; THOMSEN; MILES, 1998). Autores referem que o tecido neoplásico mamário apresenta uma maior síntese de NO e uma maior atividade da eNOS quando comparado ao tecido mamário sadio, cuja capacidade de síntese de NO é muito baixa ou imperceptível (THOMSEN et al., 1995). A atividade da NOS foi detectada apenas

em tumores de mama do tipo invasivo; o mesmo grupo demonstrou que para carcinomas ductais invasivos, a síntese de NO foi correlacionada positivamente ao grau histológico do tumor (THOMSEN et al., 1995).

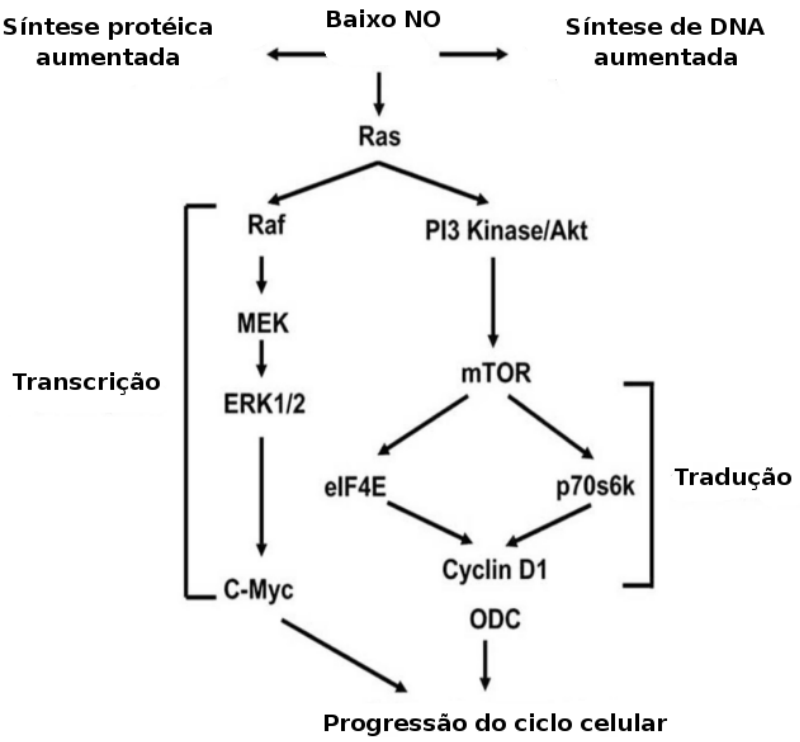
O NO é particularmente nocivo no tecido adiposo da mama, onde inicia e promove a tumorigênese. Estudos epidemiológicos associam populações a um risco maior de desenvolver o câncer de mama (especialmente em tumores ER positivos) quando apresentam polimorfismos da eNOS responsáveis pela produção diminuída de NO (PERVIN; CHAUDHURI; SINGH, 2010).

Um dos mecanismos mais conhecidos relacionados aos efeitos da eNOS na progressão do câncer de mama é a habilidade de promover a angiogênese (JADESKI; LALA, 1999; JADESKI et al., 2000; HARRIS et al., 2002). Em tumores mamários, a angiogênese pode ser estimulada por inflamação, que induz proliferação e morfogênese de células vasculares endoteliais, em resposta a uma gama de citocinas ou moléculas angiogênicas produzidas pelo tumor nas células doentes. Estudos em ratos fêmea C3H/Hej demonstraram que o NO é um mediador importante da angiogênese em tumores mamários C3L5 e os efeitos anti-tumorais de L-NAME, um conhecido inibidor de eNOS, são parcialmente mediados por uma redução da angiogênese tumoral (JADESKI; LALA, 1999; JADESKI et al., 2000). Estudos também demonstram que a caviolina-1 (CAV1), molécula que se liga e inibe eNOS, e a cavtratina, que é um peptídeo relacionado à permeabilidade celular, derivado da CAV1, prejudicam o fluxo de sangue dependente de eNOS, reduzindo bastante o crescimento do tumor (LIN et al., 2007; GRATTON et al., 2003). Uma das moléculas mais importantes na angiogênese é o VEGF. Os efeitos que acentuam o crescimento tumoral mediado por VEGF são associados com a atividade aumentada de NOS e com a inibição da apoptose em carcinomas mamários humanos xenografts (HARRIS et al., 2002). A marcação com peróxido de nitrato foi aumentada em mais de 50% em carcinomas de mama invasivos analisados e correlacionados aos níveis de VEGF-C e metástases nos nódulos linfáticos (NAKAMURA, 2006). Um estudo em ratos iNOS+/+, iNOS-/- e eNOS-/- demonstrou que na ausência da expressão de eNOS não se desenvolve o processo angiogênico. Por outro lado, nos ratos iNOS+/+ e iNOS-/- o VEGF mediou a angiogênese e a permeabilidade vascular (FUKUMURA et al., 2001). Através do estudo em culturas celulares in vitro, observou-se que o VEGF, através de feedback positivo, aumenta a fosforilação de eNOS e atividade via PI3-K/AKT, junto com a indução do fluxo de cálcio e recrutamento da proteína *heat shock 90* (HSP90) (GÉLINAS et al., 2002). A importância do VEGF na angiogênese é ampliada pela presença de vários polimorfismos, onde a associação dos fenótipos VEGF-2578A e -1498C, quando acontecem em combinação com eNOS -786 TT e eNOS 894 GG, são preditores importantes para o desenvolvimento do câncer de mama (SCHNEIDER et al., 2006; SCHNEIDER et al., 2007).

O NO está associado ao aumento da proliferação celular em câncer de mama pois

promove uma modulação de alvos e vias celulares importantes (PERVIN et al., 2007). O *RAS*, um oncogene importante, cuja ativação é capaz de elevar a sinalização das cascatas PI-3 quinase/Akt and RAF/MEK/ERK1/2, é um alvo crítico em baixos níveis de eNOS em linhagens células de câncer de mama (PERVIN et al., 2007). A hiper expressão de ERK e AKT e a desregulação de vias de proliferação e síntese proteica são bem associadas a patologia do câncer de mama (UMEMURA et al., 2007; LIU; YU; SHAIKH, 2008). Os níveis da mTOR e a quinase p70S6, que são reguladores críticos da transcrição, são um fortemente modulados por baixos níveis de NO (PERVIN et al., 2007). Também foi observado que o tratamento das células de câncer de mama com baixos níveis de NO aumentaram a absorção de timidina, caracterizando um indicador direto da síntese de DNA (PERVIN; SINGH; CHAUDHURI, 2008). Baixas concentrações de NO aumentam a proliferação de células de câncer de mama, além de aumentar a síntese proteica e de DNA, e ainda aumentam a progressão do ciclo celular da fase G1/S através do aumento dos níveis da ciclina D1 e da ornitina descarboxilase (ODC), ambos hiper expressos no tumor mamário (Figura 6) (ROY; THOMPSON, 2006; DENG et al., 2008).

Figura 6 – Baixas concentrações de NO induzem proliferação das células tumorais por meio da ativação das vias RAS/RAF/MEK/ERK e PI3 quinase/Akt, levando à ativação de mTOR, promovendo a progressão do ciclo celular e proliferação das células doentes, através da indução da ciclina D1 e ODC. O Fator eucariótico de iniciação translacional eIF4E e a quinase p70s6 são alvos finais de mTOR.



Fonte – Adaptado de Pervin, Chaudhuri e Singh (2010).

Em células MCF-7, baixos níveis de NO estão associados ao aumento da atividade de ERK através de um mecanismo dependente de cGMP (FALCONE et al., 2002). Estas concentrações de NO foram também associadas com a ativação de AKT e estabilização do fator de indução da hipóxia-1α (HIF-1α), que é hiper expresso no câncer de mama (THOMPSON; EASTON, 2004). Nas células MCF-7, a eNOS tem sido relacionada à inibição da apoptose, aumento da capacidade de sobrevivência e de proliferação celular (ÖKTEM et al., 2006). A S-nitrosilação da caspase induzida pelo NO, pode produzir células resistentes à apoptose e o acúmulo de mutações e subsequente seleção clonal. (RADISAVLJEVIC, 2004).

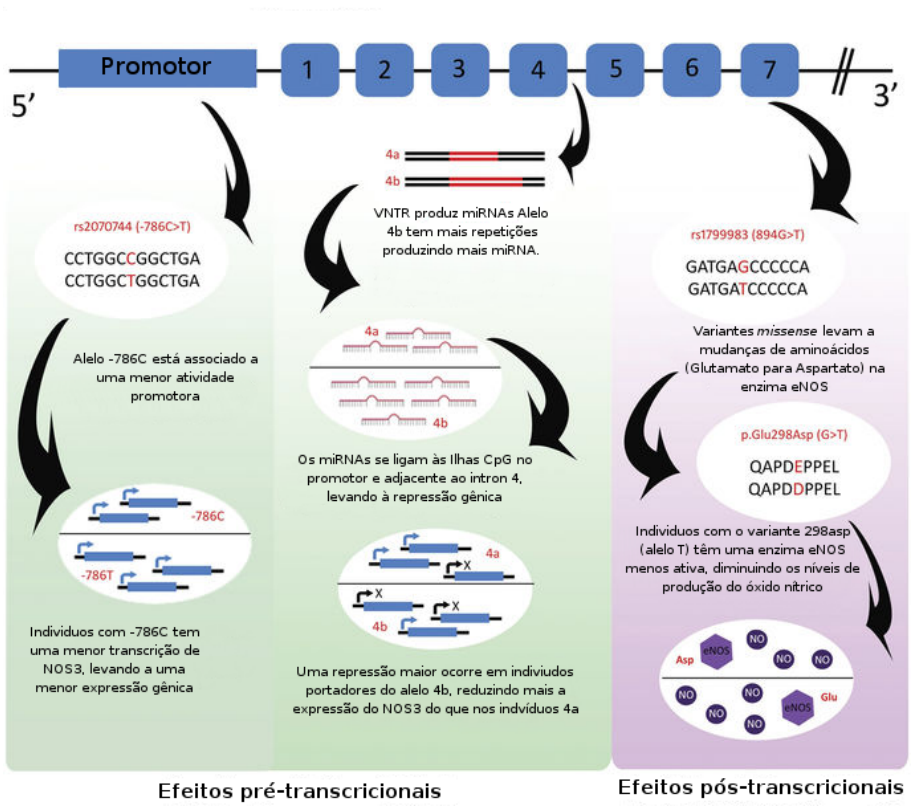
Estudos relacionados à possibilidade de associação entre os polimorfismos do gene eNOS e a susceptibilidade da mulher ao câncer de mama, produziram resultados contraditórios (HEFLER et al., 2006). Estudos epidemiológicos indicam que eNOS pode ter um papel predominante na progressão tumoral, identificando populações que apresentam polimorfismos específicos neste gene, tendo um grande risco de desenvolver o câncer de mama (TANUS-SANTOS; DESAI; FLOCKHART, 2001; LU et al., 2006; MARRONI et al., 2005; ROYO et al., 2006; MORENO, 2007). Concentrações mais baixas de NO estão associadas à presença de polimorfismos em eNOS, devido ao aumento da degradação da proteína (HEFLER et al., 2006).

O NOS3 exibe 3 variantes clinicamente relevantes, que são c.813C>T, também conhecido como g.-786T>C (rs2070744) na região promotora, o número variado de repetição em tandem (VNTR) no intron 4 e c.G894T (rs1799983) no exon 7 (Figura 7). Estes polimorfismos estão associados à modificação da expressão da eNOS uma vez que a atividade transcricional chega a ser reduzida em 50% no polimorfismo T-786C, inibindo a síntese de mRNA e a diminuição da expressão da proteína (NAKAYAMA et al., 1999).

Estudos demonstram que os variantes Asp-298 e C (-786) foram mais comuns em mulheres brancas enquanto que VNTR 4a apresentou-se significativamente maior entre a população negra (TANUS-SANTOS; DESAI; FLOCKHART, 2001; LU et al., 2006; MARRONI et al., 2005).

A consequência funcional do polimorfismo c.813C>T está relacionada à replicação da proteína de replicação A1 (RPA-1), um repressor transcricional, que se liga ao promotor do gene NOS3 com mais afinidade quando o alelo C está presente, levando à expressão diminuída do gene NOS3 e consequente diminuição da concentração de NO. A VNTR 4b/4a está associada à formação de um sirRNA. Células endoteliais que contêm o alelo 4b (5 cópias de 27bp) apresentam níveis maiores de sirRNA, o que leva a uma diminuição na expressão de NOS3 quando em comparação às células que contêm o alelo 4a (4 cópias de 27bp). O polimorfismo Glu298Asp corresponde a uma substituição Guanina (G) por uma Timina (T) na posição 894 de NOS3. Esta substituição leva a uma diminuição na ligação de NOS3 ao cavéolo da célula endotelial, resultando em menos eNOS disponível para a

Figura 7 – Mecanismos moleculares que influenciam a disponibilidade de NO.



Fonte – Adaptado de Kowalski et al. (2016).

ativação pela calmodulina, resultando, por sua vez, em uma menor atividade e produção do NO. A eNOS inativa precisa entrar no cavéolo para sua ativação. Há uma evidência de que o alelo portador de Asp modifica as interações da enzima eNOS com outras proteínas, reduzindo assim a entrada de eNOS inativa ao cavéolo, diminuindo a ativação da enzima (ZHANG et al., 2017).

Associações entre os polimorfismos de eNOS e o câncer de mama vêm sendo amplamente investigadas, no entanto os resultados desses estudos foram inconclusivos. A associação entre os polimorfismos da eNOS (intron 4a/b, T-786C e G894T) e os riscos de desenvolver o câncer de mama permanecem indefinidos. Porém, alguns estudos em G894T apontam para um maior risco de desenvolver o câncer em pacientes portadores do alelo T (HOWE; ANTHONY, 2004; LIN et al., 2014). Observou-se ainda uma significância no G894T com maior risco de câncer em mulheres em pré-menopausa que carregam o alelo T do que as portadoras do genótipo selvagem GG (HOWE; ANTHONY, 2004; MACDONALD; TAMAI; HE, 2009; IP et al., 2012).

2.6 FATOR DE TRANSCRIÇÃO TCF7L2

A via Wnt é considerada umas das mais importantes na regulação do desenvolvimento, da diferenciação celular, além de estar diretamente associada ao câncer (ZHAN; RINDTORFF; BOUTROS, 2016). Esta ligação ao desenvolvimento do câncer foi estabelecida quando houve a descoberta de int1 (Wnt1), que mediante uma inserção de um provírus em seu locus ou expressão transgênica em ratos, resultaram em hiperplasias e tumores mamários (TSUKAMOTO et al., 1988; NUSSE et al., 1984; NUSSE; VARMUS, 1982)

No início da década de 90, foram observadas mutações no gene *Adenomatous polyposis coli* (*APC*) como uma causa primária da síndrome do câncer de cólon hereditário conhecido como *adenomatous polyposis familiar* (KINZLER et al., 1991; NISHISHO et al., 1991). A correlação do gene *APC* com a β-catenina (β-cat) foi inicialmente observada através da perda de função do *APC*, que resultava em uma superexpressão *T-cell factor (TCF) 4/β-catenin signaling*. Estas observações permitiram a ligação entre a via Wnt e o câncer de cólon (RUBINFELD et al., 1993; SU; VOGELSTEIN; KINZLER, 1993; KORINEK, 1997).

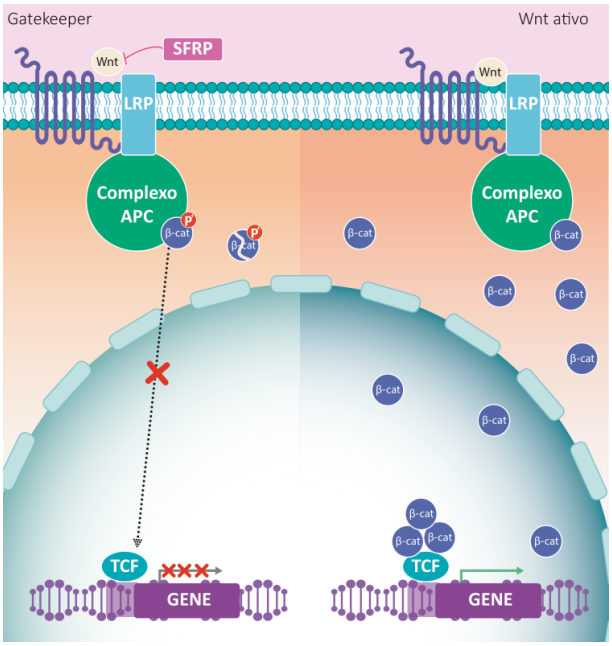
A via Wnt é ativada em mais de 50% dos pacientes de câncer de mama e está ligada à uma taxa de sobrevivência reduzida (LIN et al., 2000). O papel da via Wnt no desenvolvimento e progressão do câncer de mama triplo negativo (TNBC) foi amplamente estudado (GEYER et al., 2010; KHRAMTSOV et al., 2010; XU et al., 2015). Apenas uma pequena fração de tumores apresentam mutações somáticas de vias metabólicas importantes como é o caso da β-cat, mas os ligantes desta via e os receptores na via Wnt estão comumente superexpressos no câncer de mama, enquanto os antagonistas são silenciados (HOWE; ANTHONY, 2004; LIU et al., 2010; YANG et al., 2011; KLARMANN; DECKER; FARRAR, 2008).

Quando a expressão da β-cat está aumentada, esta se transloca para o núcleo e se liga ao TCF7L2 (fator de transcrição 7 tipo 2) formando um complexo nuclear, que ativa genes importantes em várias funções celulares normais como a regulação do crescimento. O gene TCF7L2 ou Fator celular 4 (Tcf-4) está localizado no cromossomo 10q25.3, com 215.9 kb, formado por 17 exons e codifica um fator de transcrição de alta mobilidade (MISHRA; VERMA, 2010). Esta molécula faz parte da via de sinalização Wnt/β-catenin (Figura 8) e atua de forma crítica na regulação do crescimento celular (CHEN et al., 2013). O TCF7L2 está bem descrito em relação a diabetes e obesidade (MISHRA; VERMA, 2010). Além disso, ele pode influenciar o câncer de forma independente em diabéticos por ser um componente da via Wnt/β-cat e exercer funções de regulação no desenvolvimento celular (MIN et al., 2016; INCA, 2018).

A β-catenina se liga ao TCF7L2 no núcleo, ativando o fator de transcrição. Este

evento resulta na expressão de oncogenes específicos como ciclina D1, MCP-1, c-myc, que são característicos nos cânceres humanos (WALSH et al., 2016; CATTEAU et al., 1999; TOSS; CRISTOFANILLI, 2015; PRAT; ELLIS; PEROU, 2011). Estudos prévios apontam uma super expressão do TCF7L2 em carcinoma hepatocelular, câncer de mama, carcinoma de esôfago e sua expressão está correlacionada à progressão da doença (HORTOBAGYI, 1994; WEIGELT; PETERSE; VAN'T VEER, 2005; EIFEL et al., 2001; KAZANETS et al., 2016; TIMP; FEINBERG, 2013; MIN et al., 2016).

Figura 8 – Eventos de silenciamento gênico na via Wnt.



Fonte – Adaptado de Baylin e Ohm (2006).

Em células sadias, os níveis de β-cat livres na célula são mantidas por meio da sua degradação pelo complexo APC, AXIN e GSK3. Este complexo fosforila a β-cat nos resíduos N-terminais e se liga a proteína para a inativação ou degradação (CONNOR et al., 2012; SIERAKOWSKA et al., 1993). Quando este processo não ocorre, a β-cat se transloca para o núcleo e ativa fatores de transcrição da família TCF/LEF1, formando um complexo que estimula a expressão de genes como cyclin D1 e c-myc (CONNOR et al., 2012) e estimula cascatas de sinalização, incluindo o AMP cíclico e insulina em células específicas (KAPPES et al., 2001).

Neste gene existem polimorfismos que estão fortemente associados ao risco de diabetes, no entanto, grupos de estudo têm desenvolvido meta-análises que apontam para uma relação entre os alelos variantes rs12255372 (NG_012631.1:g.103894G>T) e rs7903146 (NG_012631.1:g.53341C>T) do gene com uma maior susceptibilidade ao câncer de mama. Porém, existe a necessidade de estudos mais amplos e em larga escala que viabilizem uma

ampliação no conhecimento sobre o tema (ALEXIADIS et al., 2000).

O mecanismo inerente à associação entre TCF7L2 e o câncer de mama ainda não está bem estabelecido, mas outros genes alvo para o TCF7L2 têm sido observados para o câncer de mama, incluindo *Monocyte Chemotactic Protein-1* (MCP-1/CCL2), *RAD6B* e Osteopontina (*OPN*), que tem sido associados à metástase (DEUTZMANN et al., 2014).

2.7 MOLÉCULA DEK

DEK é uma proteína composta por 375 aminoácidos e 43KDa (LINDERN et al., 1990). A maior porção dela está associada à cromatina, o que aponta para sua importância na arquitetura do genoma humano (ABBA et al., 2007). A proteína codificada pelo gene *DEK* induz alterações na densidade, reduzindo a eficiência da capacidade de replicação da cromatina (HAN et al., 2009). Todas as funções distintas da DEK no DNA são específicas em relação à estrutura do DNA, com preferência por uma conformação condensada ou cruciforme (KROES et al., 2000).

A proteína DEK foi apresentada à comunidade científica no início da década de 90, como parte de uma translocação cromossômica (6;9)(p23;q34) em pacientes portadores de leucemia mielóide aguda (AML) (SANCHEZ-CARBAYO et al., 2003). Desde então, a DEK tem sido observada em diversos tecidos humanos por sua superexpressão em tumores de diferentes origens incluindo os de pele, mama, ovários, cerebral, bexiga, entre outros (ABBA et al., 2007; CARRO et al., 2006; WISE-DRAPER et al., 2005; JOHUNG; GOODWIN; DIMAIO, 2007; PRIVETTE VINNEDGE et al., 2012).

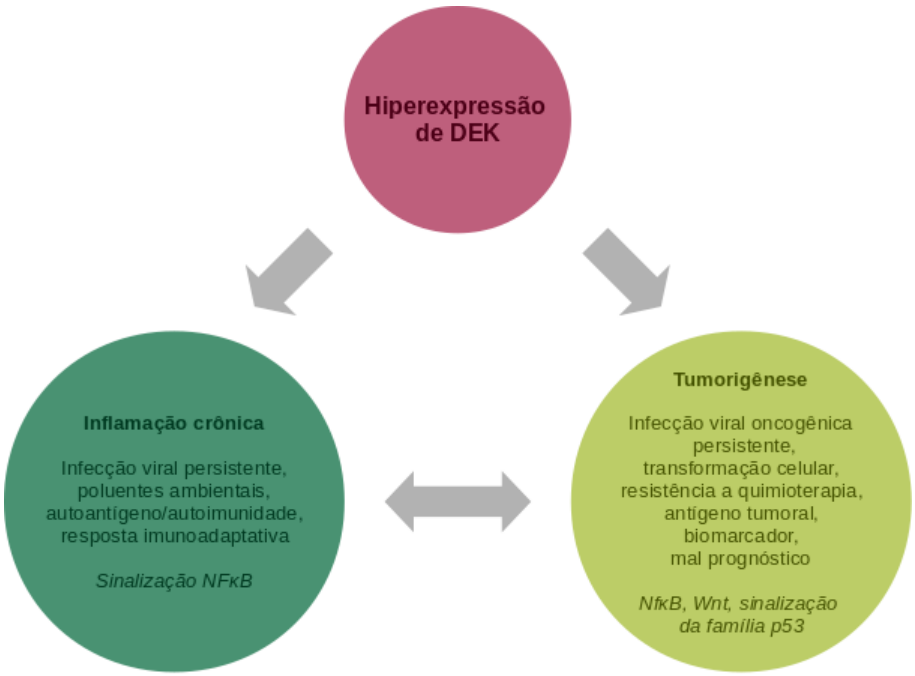
Em uma célula normal, o promotor do *DEK* é ativado por fatores transcricionais E2F, que também são fatores chave para a Proteína Retinoblastoma (pRb), que também o regula. Acredita-se que a perda de função do pRb pode levar a uma atividade anormal dos fatores de E2F, além da inativação do p53, um importante supressor tumoral. Estas são vias cruciais, que uma vez desreguladas são capazes de desestabilizar as defesas celulares contra neoplasias, o que permite uma proliferação celular aumentada (CARRO et al., 2006).

Vários fatores são capazes de induzir inflamações de forma crônica, incluindo bactérias persistentes e infecções virais, exposição a poluentes ambientais, além de doenças autoimunes. A via NFκB é crucial na inflamação e no desenvolvimento de tumores, principalmente levando-se em consideração que pacientes que sofrem de inflações crônicas apresentam um risco maior de desenvolver câncer (DIAKOS et al., 2014). As funções relacionadas à sobrevivência celular de NFκB promovem a viabilidade celular ao passo que as citocinas produzidas pela atividade transcricional do NFκB vão alterar a resposta imune anti-tumoral (HOESEL; SCHMID, 2013). A proteína DEK, dentre seus diversos papéis, promove a inflamação. Isto inclui: a indução do ciclo de vida e fase latente de

vírus oncogênicos; a expressão aumentada, mediante exposição a poluentes ambientais; ser um auto antígeno potente em doenças inflamatórias crônicas; e desempenhar um papel pró-inflamatório promovendo a migração de células brancas do sangue, secretadas por macrófagos ativados (Figura 9) (PEASE; WISE-DRAPER; PRIVETTE VINNEDGE, 2015).

No câncer cervical, a inibição da pRb pela ação da oncoproteína E7 do HPV leva à superexpressão da oncoproteína DEK. Desta forma, a superexpressão do DEK parece ser um evento comum em quadros de carcinogênese, refletindo sua função de inibição da senescência (WISE-DRAPER et al., 2005), podendo representar um novo biomarcador e potencial alvo terapêutico.

Figura 9 – Sumário dos papéis de DEK na inflamação e na tumorigênese.



Fonte – Adaptado de Pease, Wise-Draper e Privette Vinnedge (2015).

No câncer de mama, DEK favorece crescimento e invasão das células neoplásicas e manutenção de células tronco tumorais. A expressão de DEK está associada à positividade para os receptores de hormônios em células de câncer primárias, estando superexpressa *in vitro* quando expostas aos estrógenos, progesterona e andrógeno. Experimentos de imunoprecipitação de cromatina apontam DEK como um novo gene alvo dos receptores de estrógenos α (ERα), cuja expressão promove a proliferação induzida por estrógenos. Foi observado que a deleção de DEK aumenta a morte induzida pelo tamoxifeno em células de câncer de mama (ER+), demonstrando seu poder carcinogênico e seu potencial preditivo (PRIVETTE VINNEDGE et al., 2012).

3 RESULTADOS

Os resultados deste trabalho são apresentados nos artigos *eNOS role in breast cancer – an integrative review* e *NOS3 variants and TCF7L2 expression associated to breast cancer*, ainda não publicados, e no artigo *The unique DEK oncoprotein in women's health: a potential novel biomarker*, publicado no periódico *Biomedicine & Pharmacotherapy Journal*, que consta no Apêndice A.

3.1 ENOS ROLE IN BREAST CANCER – AN INTEGRATIVE REVIEW

Ana Cecília de Albuquerque Oliveira 1,*, Victor Borba 2, Danyelly Bruneska Gondim Martins 1,3, José Luiz de Lima Filho 1,3

1 - Molecular Prospecting and Bioinformatics Group – Laboratory of Immunopathology Keizo Asami (LIKA) – Federal University of Pernambuco (UFPE). Av. Prof. Moraes Rego, 1235 - Cidade Universitária, Recife - PE, Postal Code 50670-901, Brazil.

2 - University of Ottawa – 75 Laurier Ave E, Ottawa, ON K1N 6N5, Canadá

3 - Department of Biochemistry – Federal University of Pernambuco (UFPE), Brazil. Av. Prof. Moraes Rego, 1235 - Cidade Universitária, Recife - PE, Postal Code 50670-901, Brazil

*Corresponding author:

anaceciliaao@gmail.com

Av. Prof. Moraes Rego, 1235 – Cidade Universitária, Recife - PE, Brazil, CEP: 50670-901. Phone: +55 81 2126-8484

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Abstract

eNOS is an enzyme responsible for NO generation, and involved in many pathways that could contribute for cancer development and maintenance. The aim of this study was analyze the existing evidence on eNOS role in breast cancer through an integrative review. A systematic search was conducted using online database Pubmed, with "eNOS and breast cancer" as specific key words. 89 articles were found and then filtered excluding meta-analyses, reviews, unavailable articles, other themed articles. After that, inappropriate articles and polymorphic articles were excluded. NOS is detected predominantly in in situ lesions and invasive breast lesions and It is found more frequently in invasive carcinomas with low malignancy. eNOS showed to have potential as a prognostic marker and therapeutic target once it interacts with pathways involved in cell cicle division and power of migration.

Keywords: eNOS, breast cancer, VEGF, endothelial, angiogenesis.

1 Introduction

In 2012, breast cancer was the second most diagnosed cancer worldwide, with 1.67 million cases in that year, accounting for 25% of all cancer diagnoses in women [1]. The mechanism of breast cancer development is still not yet understood, though risk factors of breast cancer development are known to include being female, obese, drinking alcohol, and early age menarche. It is also known that various inherited genetic factors contribute to susceptibility of breast cancer [2].

Nitric oxide (NO), an inorganic free radical gas, is synthesized from the amino acid L-arginine by a group of enzymes, the NO synthases (NOS) [3,4]. NOS activity has been reported in human breast tumors and was found to be higher in invasive tumors compared to normal or benign tissues [5]. More recently, it has been demonstrated that patients with triple-negative breast tumors expressing NOS had a significantly worse prognosis compared to those that did not express NOS [6].

At least three isoforms of NOS have been cloned, characterized, and localized [3]. Endothelial (eNOS) and neuronal (nNOS) NOS isoforms are Ca^{2+}/cal- modulin-dependent and are expressed constitutively in these and other cells that control important physiological processes such as vasodilatation (eNOS) and neuronal transmission (nNOS) [7–9]. The inducible (iNOS) isoform of NOS is Ca^{2+} / calmodulin-independent and usually induced in the presence of inflammatory cytokines and bacterial products in macrophages, hepatocytes, and other cells. Under certain conditions, iNOS can also be expressed constitutively in some cells. When constitutively expressed, NO produced at low levels is an important mediator of physiological functions such as vasodilation, inhibition of platelet aggregation, and neurotransmission [10,11].

NO has a dichotomous activities in many areas of biology. It can promote cell survival but also has pro-apoptic effects; also, NO is able to stimulate cell growth as well as cell death and necrosis. As a potent vasodilator, NO controls the dynamic balance, but it can also promote angiogenesis, which is related to the loss of vasoactivity. NO roles have been widely exemplified, showing that the net effect of NO depends on it available concentration in a certain environment as it was revised by [12].

Several factors present in tumor microenviroment have the capacity to modulate eNOS expression. First, it has been consistently demonstrated that hypoxia down-regulates eNOS expression in cultured pulmonary human endothelial cells [13]. In non-pulmonary endothelial cells, the findings are more controversial: both increased [14] and reduced [15]. eNOS expressions have been reported using similar models of endothelial cell hypoxia. Furthermore, eNOS mRNA and eNOS protein levels are increased in proliferating cells compared to resting endothelial cells [16]. Third, cytokines can regulate eNOS expression in different ways depending on the cytokine combination, the animal species, and the cell type. For example, lipopolysaccharide (LPS) injection in rats reduces eNOS mRNA expression in the aorta [17]; by contrast, it increases mRNA abundance in cultured bovine endothelial cells [18]. Finally, growth factors may up-regulate eNOS mRNA levels. Incubation of endothelial cells with transforming growth factor (TGF)-β enhances the eNOS promoter activity. [19]. And VEGF has been described to increase eNOS expression via the VEGF receptor-2 (VEGFR-2) pathway in cultured human endothelial cells [20].

Tumor angiogenesis initiates with vasodilation, a process involving VEGF-eNOS release [21]. Subsequently, vascular permeability increases in response to VEGF, which implicates the formation of vesicular organelles, partly through the coalescence of caveolae. Interestingly, VEGF-dependent, Akt-mediated increase in vascular permeability is inhibited by the NOS inhibitor L-NAME, indicating that NO could play a crucial role in the early steps of tumor angiogenesis [22].

The regulation of oxidative stress is an important factor in both tumor development and responses to anticancer therapies. Many signalling pathways that are linked to tumorigenesis can also regulate the metabolism of reactive oxygen species (ROS). Cancer cells are characterized by increased aerobic

glycolysis (termed the Warburg effect) and high levels of oxidative stress exerted by ROS. The high ROS levels in cancer cells are a consequence of alterations in several signalling pathways, and eNOS is one of the enzymes involved [23].

Increased expression of eNOS has been noted in the vasculature of various tumor tissues, including bladder, colon, and pancreatic cancers [2,24,25]. Previous studies have shown that eNOS can modulate cancer-related events, such as angiogenesis, invasion, and metastasis [26–28]. eNOS is a central mediator of several endothelium growth stimulators, such as VEGF and prostaglandin E2. VEGF can increase angiogenesis in absence of iNOS but not without eNOS, suggesting a predominant role of eNOS in VEGF-induced angiogenesis [26]. In addition, an in vivo study has indicated that high eNOS expression is correlated to trophoblast cancer cell vascular invasion [27]. Tumor cells in lung metastatic sites are always strongly eNOS-positive, suggesting that eNOS expression facilitates metastasis [28].

In this estudy we performed an integrative review about the role of eNOS expression in breast cancer.

2 Materials and methods

The keywords *eNOS* and *breast cancer* were used in PubMed open database search engine [29] in March 2018. The resulting articles were further filtered to include only original research articles and in English.

The meta-analyses, reviews, other types of cancers papers were excluded (Figure 1). The polymorphic analyses were also excluded, since it was out of the focus of this work.

Investigating if the expression of eNOS molecules involved in breast cancer was the principal aspect in this work.

3 Results and discussions

The results from the criteria of inclusion showed that from a total of 89 articles, 69 were excluded and 20 were deemed appropriate to further evaluation.

eNOS is expressed by human breast tumors, in endothelial cells, and its presence negatively correlates with histologic grade and lymph node status and positively correlates with Estrogen Receptor expression [30–32].

In breast cancer cases, the major molecules (HIF1-α, VEGFR-2) regulating NO and VEGF production can be co-expressed in the individual carcinomas implying a possibility for the relevant pathways to be active like PI3K, PKC [33]. A dual role for NO, either inductive or inhibitory, has been proposed on the basis of different effects that high or low concentrations of NO may exert on the angiogenic process [34]. Its synthesis might be regulated by hormonal stimulation since eNOS expression is also observed associated with the positive estrogen and progesterone receptors [35,36]. Loss of NOS expression may be associated with the progression of breast cancers. [37,38].

NOS is detected predominantly in in situ lesions and invasive breast lesions but rarely in benign lesions. It is found more frequently in invasive carcinomas with low malignancy [39].

In phyllode tumors, which are non-malignant tumors, NO is associated with malignancy, which suggests a possible role in malignant progression, with metastatic potential [6].

Tumor cells prefer to adhere to the microvessel locations with a higher NO production such as curved portions, suggesting that inhibition of eNOS could be a good approach to preventing tumor cell adhesion to intact microvessels under physiological flows, which could be an alternative to repress malignancy of a mutated cell in a less malignant situation [40].

The secretion of matrix metalloproteinases (MMPs) in MCF-7 cells is under control of NO by eNOS mRNA levels. MMPs are proteolytic enzymes involved in the degradation of extracellular matrix,

probably related to eNOS action in cell migration. These degradation is related to the inicial uncontrolled spread of proliferating cancer cells and therefore plays a crucial role in cancer invasion and metastasis. Gach et al.[41] observed that morphine and EM-2, MMP modulators, are able to decreased endothelial nitric oxide synthase.

In patients with metastatic breast cancer, the antiangiogenic properties of weekly docetaxel showed that low eNOS mRNA levels have adverse prognostic significance for overall survival independently from established clinical prognostic factors [42].

The research related to regulation of eNOS bring some directions in crucial controlling pathways in cell malignancy. Several cationic amino acid transporter (CAT) isoforms are expressed in breast cancer cells and plays an important step in cell survival. It is responsible for arginine uptake in growth of the human MCF-7 breast cancer cell line. L- arginine is released from extracellular substrates by prolactin (PRL)- and 17β-estradiol (E2)-induced carboxypeptidase-D in the cell membrane, promotes NO production for MCF-7 cell survival [43].

In breast cancer, overexpression of Heat shock protein 90 (Hsp90) is correlated with poor prognosis [44]. Hsp90-dependent phosphorylation of eNOS is a critical event that determines eNOS activity [45]. Hsp90, is a molecular chaperone, that associates with eNOS to accelerate its catalytic activity by creating a favorable biological environment for AKT-dependent phosphorylation. Thus, Hsp90/eNOS association influences the rate of AKT-dependent phosphorylation, unmasking the phosphorylation sites on eNOS and maintaining the p-AKT levels by inhibition of proteasomal-mediated degradation of PI3K [46–48].

The AKT signaling network plays a functional role in endothelial cells, vascular smooth muscle cells, and macrophages. AKT1 appears to be the major isoform that contributes to normal endothelial cell physiological functions. It was observed that activation of AKT1 by VEGF stimulates cell proliferation, migration, and survival [49]. eNOS is an AKT1-specific substrate, and in PI3K pathway the phosphorylation of NOS through AKT promotes nitrosylation and activation of wild-type Ras proteins and the MAPK wish is key pathway in tumorigenesis [26]. Two approved anticancer drugs, everolimus and temsirolimus, exemplify targeted inhibition of PI3K/AKT/mTOR in the clinic and many others are in preclinical development as well as being tested in early clinical trials for many different types of cancer [50].

ELK1, a transcription regulating factor downstream MAPK pathway, was also found activating eNOS and controlling growth signaling in prostate, breast, and among other cancers [51].

ESR1, and estrogen related protein, is first activated by cytoplasmic estradiol, and has an influence on expression and activation of eNOS. Specifically, it is present in high quantities for two thirds of breast cancer cases [52]. Nevertheless, a long cascade activates ESR2, a transcription factor that activates eNOS, and its variants result in increased breast cancer risk [53]. It has also been shown that both, eNOS and ESR2 together, are in high levels for prostate cancer [54], suggesting a role in cancer cells. eNOS produces intracellular NO that activates Guanylate Cyclase [55] and, through cyclic GMP, can activate MAPK and B-cell lymphoma 2 (BCL2) inducing or inhibiting apoptosis [56]. This pathway suppresses apoptosis since BCL2 binds to the IP3 ligand gated ion channel [57], avoiding the full Ca2+-dependent signalling important for cell growth [58].

All the following pathways start with the VEGF growth factor receptor ligand binding to SP3, CREB1, and AP1. SP3 is a transcription regulation activator associated with breast cancer propagation [59], while CREB stands for cAMP, a transcription regulator that activates eNOS. This protein is related to breast cancer [60], as well as AP-1, which is also a transcription regulator factor complex that activates eNOS [61].

Caveolin-1 (CAV-1) is a tumor suppressor that is inhibitory to eNOS. It has been shown that a loss of CAV creates an environment where breast cancer can strive [62], due to the oxidative stress created environment since eNOS is inhibited, reducing the NO concentration necessary to neutralize reactive

oxidative substances. eNOS-expressing fibroblasts have the ability to downregulate CAV-1 and induce mitochondrial dysfunction in adjacent fibroblasts that do not express eNOS. As such, the effects of stromal oxidative stress can be laterally propagated, amplified and are effectively contagious spread from cell-to-cell like a virus, creating an *oncogenic/mutagenic* field promoting widespread DNA damage [63].

The silencing of CAV-1 promotes PI3K and AKT pathway activation, so Cavtratin, a derivative of CAV-1, is able to inhibit tumor progression by blocking microvascular permeability [64]. Cavtratin activates the CAV-1 pathway, which is inhibitory to eNOS, working as a tumor suppressor, therefore the whole cascade leading eNOS will be halted. This protein has not had further research done as a drug, but the pathway could be promising as a therapeutic target [50].

There are other molecules that can activate or inhibit eNOS, but the mechanism of action is still unknown. Other molecules like KLF2 (Kruppel-like Factor), a transcription factor, was observed as silenced in breast cancer cells [65]; SP1, that is correlated to gastric cancer [66]; and PKC-beta that shows to promote oral cancer metastasis [67], could also plays a role in eNOS activation and breast cancer initiation and maintenance, although not yet studied in this type of neoplasm.

There are also proteins that inhibit eNOS in these pathways: caveolin-3 that binds to eNOS, and if caveolin-3 does not express itself, breast cancer develops [68]; (ii) bradykinin receptor (BDKRB2) also inhibits eNOS and has been shown to be related with bladder cancer [69], and (iii) endothylin B receptor (EDNRB), which is a G-protein coupled receptor (GPCR) that conducts inhibition on eNOS, and relation with skin cancer [70].

4 Conclusion

Overall, this study suggests that there is a strong link between eNOS and cancer. There is a multitude of proteins that are both incorporated into eNOS pathways as well as being correlated with different types of cancer, particularly breast cancer. Most notably, ER activation is related to eNOs concentrations and VEGFR in PI3K-AKT pathway are the most cited (6 articles), which suggest eNOS as an important target for therapeutic target in preventing tumor cell adhesion and migration.

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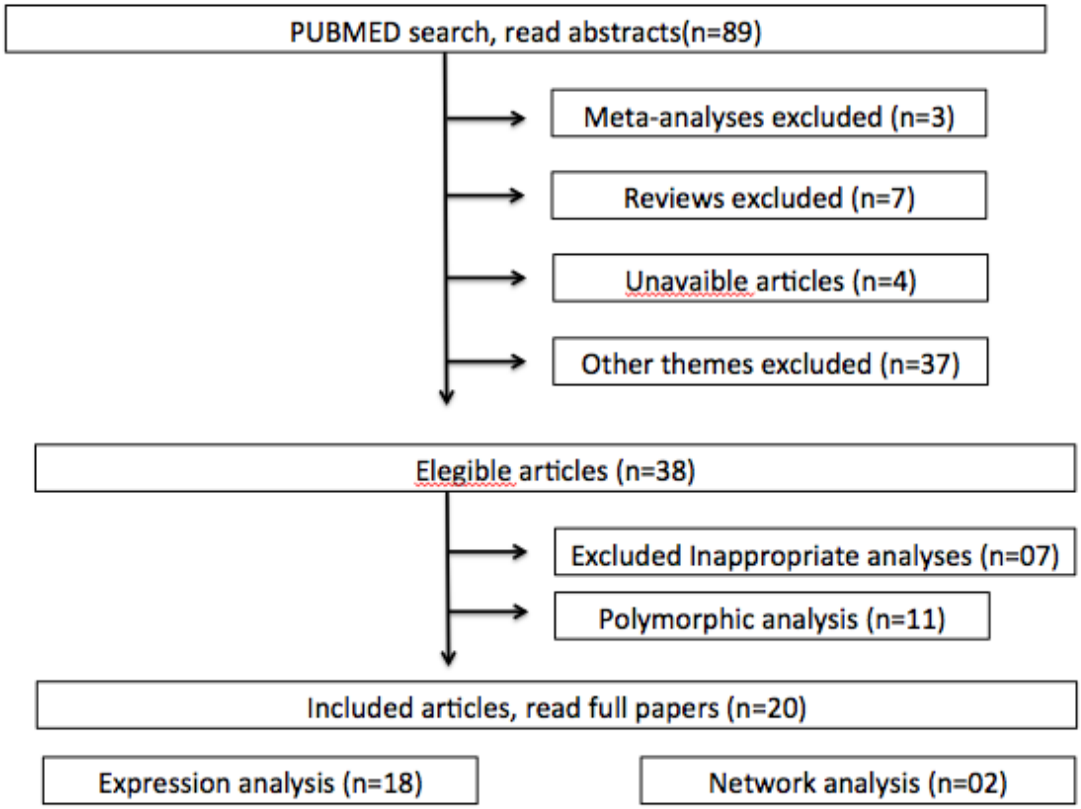
Figure Legends

Figure 1. Meta-analysis workflow.


PUBMED search, read abstracts(n=89)
Meta-analyses excluded (n=3)
Reviews excluded (n=7)
Unavaible articles (n=4)
Other themes excluded (n=37)
Elegible articles (n=38)
Excluded Inappropriate analyses (n=07)
Polymorphic analysis (n=11)
Included articles, read full papers (n=20)
Expression analysis (n=18)
Network analysis (n=02)

3.2 *NOS3* VARIANTS AND *TCF7L2* EXPRESSION ASSOCIATED TO BREAST CANCER

Ana Cecília de Albuquerque Oliveira 1,*, Victor Borba 2, Andriu dos Santos Catena 1, Danyelly Bruneska Gondim Martins 1,3, José Luiz de Lima Filho 1,3

1 - Molecular Prospecting and Bioinformatics Group – Laboratory of Immunopathology Keizo Asami (LIKA) – Federal University of Pernambuco (UFPE). Av. Prof. Moraes Rego, 1235 - Cidade Universitária, Recife - PE, Postal Code 50670-901, Brazil.

2 - University of Ottawa – 75 Laurier Ave E, Ottawa, ON K1N 6N5, Canadá

3 - Department of Biochemistry – Federal University of Pernambuco (UFPE), Brazil. Av. Prof. Moraes Rego, 1235 - Cidade Universitária, Recife - PE, Postal Code 50670-901, Brazil

*Corresponding author:

anaceciliaao@gmail.com

Av. Prof. Moraes Rego, 1235 – Cidade Universitária, Recife - PE, Brazil, CEP: 50670-901. Phone: +55 81 2126-8484

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Abstract

Nitric Oxide (NO) is a short-lived and small molecule that acts as a biological mediator with anti-tumoral activities, which can be synthetized by Nitric Oxide Synthase (*NOS3*). NO inhibits the transcriptional activity of β-catenin, so its interaction with TCF7L2 and activation of genes involved in cell proliferation, differentiation and survival. The aim of this study was to evaluate the influence of NOS3 T (-786) C (rs2070744) in the promoter, the VNTR in intron 4 (rs1722009) and Glu298Asp (rs1799983) variant in exon 7 and *TCF7L2* g.49080T>C, (rs7901695), g.103894G>T (rs12255372), g.53341C>T (rs7903146) variants in breast cancer patients. Gene expression levels were also analyzed in this group. *NOS3* c.-813C>T showed significance for history of familial cancer in allele C and *TCF7L2* expression as significance for overweight patients. Our results suggest that the allele C of c.-813C>T in *NOS3* are involved in familial cancer; and *TCF7L2* expression can be related to overweight in breast cancer patients. Further analysis should be performed to determine the influence of these in breast cancer, but based in literature and with the association with familial cancer, eNOS can be pointed as the best particle for prognosis if compared with TCF7L2.

Keywords: eNOS, T-786C, Breast cancer, Intron 4, TCF7L2.

1 Introduction

The understanding of the molecular mechanisms that give rise to breast tumors is incomplete. It is now accepted that breast cancer is not a single disease, but instead it is composed of a spectrum of tumor subtypes with distinct cellular origins, somatic changes, and etiologies [1].

Endothelial cells are a major component of the tumor microenvironment stromal compartment, critical for the development and progression of primary breast cancer. Nitric Oxide (NO), which can be synthetized by Endothelial Nitric Oxide synthase (eNOS) acts as a biological mediator in anti-tumoral activities through inhibition of protein kinase B (AKT) pathway phosphorylation [2].

The specific role of *NOS3* expression and the risk of propagation of developing breast cancer is currently uncertain. The variants T-786C (rs2070744) (indiviuals with -786C allele have less NOS3 transcription leding to less gene expression); c.G894T (rs1799983) (Individuals with 298Asp variant, coded by allele T, have an eNOS enzyme less active, changing the levels os nitric oxide production) and Intron 4a/b (rs1722009) (produces miRNA, allele 4b has more repetitions, then produces more miRNAs, reducing NOS3 expression) are the most studied for their associations with cancer risk [3–6]. However, the relationship between eNOS polymorphisms and breast cancer has conflicting results.

It is known that NO inhibits the transcriptional activity of β-catenin, avoiding its translocation to the nucleus, interaction with T-cell factor/lymphoid enhancer factor (TCF/LEF) and activation of genes involved in cell proliferation, differentiation, cell fate and survival. This mechanism modulates the activation of Wnt/β-catenin pathway, involved in the expression of vascular endothelial growth factor (VEGF) [2,7]. Aberrant Wnt signalling contributes to the pathogenesis of many cancers, and TCF7L2 (also known as TCF4) is the most intensely studied member of the TCF/LEF family. *TCF7L2* has been studied for colorectal cancer [8,9], but seems to be also associated with breast cancer risk [10].

In this study, it was performed an evaluation of *eNOS* and *TCF7L2* variants and expression in breast cancer patients.

2 Materials and methods

2.1 Samples

Breast tissue samples from 27 patients were collected during to the breast surgery at Department of Mastology from Barão de Lucena Hospital, in Recife – Brazil. Samples were stored in RNAlater (ThermoFisher Scientific, USA) until DNA and RNA purification. The information about physiological status like age, smoking status, number of pregnancies, menopausal, cancer grade and stage and other characteristics were obtained from a questionnaire (Table 1). This study was approved in the Ethical Committee of Federal University of Pernambuco and informed consent was obtained from all patients.

2.2 Nucleic acid purification and preparation

DNA and RNA were obtained from tissues fragment using automated QIAsymphony instrument (Qiagen, USA). After extraction, DNA and RNA concentration and purify were evaluated in NanoDrop^{TM} Spectrophotometer (Thermo Scientific, USA). cDNA was obtained using QuantiTect Reverse Transcription Kit (Qiagen, USA), following manufacturer's instructions. DNA, RNA and cDNA were stored at -80^{o}C until processing.

2.3 *NOS3* variants determination

The *NOS3* Intron 4 VNTR a/b was evaluated through PCR, while c.-813C>T (NC_000007.14:g.150992991C>T, also known as T-786C or -786T>C) (rs2070744) and c.894T>G (rs1799983) variants were evaluated through RFLP-PCR using GoTaq Green Master Mix (Promega, USA), following the manufacturer's protocol.

The VNTR (27bp repeat) in intron 4 was detected by PCR using primers Fw 5'-AGG CCC TAT GGT AGT GCC TTT-3' and Rv 5'-TCT CTT AGT GCT GTG GTC AC-3' at 63°C annealing step. The PCR final volume was 25μl: 12.5 μl od GoTaq Green Master Mix (Promega), 1.0 μl of DNA, 0.8 μl od primers sense and antisense. The denaturation was 95°C for 2', tha amplification condition was 35 cicles of variate temperatures to DNTPs annealing (95°C for 30", 64°C for 30", 72°C for 1") and a extension cicle of 5 minutes of 72°C. The following fragments were 393 bp (4 copies of the 27 bp - allele a), and 420 bp (5 copies of the 27 bp – allele b). The amplified product was size-fractionated by electrophoretic separation on 2.5% agarose gel stained with ethidium bromide.

The -786T>C polymorphism in the 5' flanking region of *NOS3* was evaluated using the primers Fw 5'-TGG AGA GTG CTG GTG TAC CCC A-3' and Rv 5'-GCC TCC ACC CCC ACC CTG TC-3' at 65°C annealing step. The PCR final volume was 25μl: 12,5 μl od GoTaq Green Master Mix (Promega), 1.0 μl of DNA, 0.8 μl od primers sense and antisense. The denaturation was 95°C for 2' the amplification condition was 35 cicles of variate temperatures to DNTPs annealing (95°C for 30", 66°C for 30", 72°C for 1") and a extension cicle of 5 minutes of 72°C. The amplicon was treated with MspI at 37°C for 3 hours, and the products were fragments of 140/40 bp for the allele T; and 90/50/40 bp for allele C. The fragments were observed on 2% agarose gel stained with ethidium bromide.

The *NOS3* c.894G>T variant in exon 7 was determined using primers Fw 5'-AAG GCA GGA GAC AGT GGA TGG A-3' and Rv 5'-CCC AGT CAA TCC CTT TGG TGC TCA-3' at 65°C annealing step. The PCR conditions were the same for -786T>C polymorphism. The 248 bp fragment was treated with the BanII at 37°C for 1 hour, resulting in 163/85bp for allele G or no digestion for allele T. The fragments were observed on 2% agarose gel stained with ethidium bromide [11].

2.4 *TCF7L2* variants determination

Three *TCF7L2* variants were evaluated using fluorescent probes (TaqMan©, Life Technologies, USA): g.49080T>C (rs7901695, Life ID - C_384583_10); g.103894G>T (rs12255372, Life ID: C_291484_20); and g.53341C>T (rs7903146, Life ID: C_29347861_10). Genotyping reactions were performed in duplicate using TaqMan® Universal PCR Master Mix (Life Technologies, USA), according to manufacturer's guidelines. DNA amplification and allelic discrimination plot were performed in StepOnePlus™ System (Life Technologies, USA).

2.5 *NOS3* and *TCF7L2* expression

The *NOS3* and *TCF7L2* expression analysis was performed in duplicate using Rotor-Gene SYBR® Green PCR Kit (Qiagen©) in RotorGene Q (Qiagen©), according to manufacturer's guidelines. *NOS3* commercial primer set (Hs.PT.58.21447620 – IDTDNA, USA) was used for gene expression analysis. The sequences for *TCF7L2* primers were Fw 5'-CAC ACT TAC CAG CCG ACG TA-3' and Rv 5'-TCC TGT CGT GAT TGG GTA CA-3'. RPLP0 gene was used as housekeeping gene Fw 5'-TCT ACA ACC CTG AAG TGC TTG ATA TC-3' and Rv 5'-GCA GAC AGA CAC TGG CAA CAT T-3'. Samples with poorly preserved RNA were excluded based on preliminary results for reference gene.

2.6 Statistical analysis

NOS3 and *TCF7L2* genotype distributions were tested for adherence to the Hardy–Weinberg equilibrium by performing the χ^2 test. Body mass index (BMI, kg/m^2) at diagnosis was categorized based on WHO criteria (normal: 18.50–24.99 kg/m^2; overweight: 25.0–29.9 kg/m^2; obese: >30 kg/m^2). Data analysis was performed in Prism 6.0 software (GraphPad software, USA) and p value ($p<0.05$ was considered significant).

3 Results

All genotypes were in Hardy–Weinberg equilibrium. A total of 22 breast cancer patients were included in this evaluation, enrolled in this study had on average 54 years old at the diagnosis varying from 28 to 100 years, 40.9% of them were in menopause, while 20.0% had smoke history. The predominant histological classification was Infiltrating Ductal Carcinoma (ICD) representing 86.8%; the Luminal subtype was the most prevalent according to Immunohistochemistry (68.2%). Most of the cases were diagnosed in late stages, 40.9% of the cases were diagnosed in stage III, with the size of the primary tumors were larger than 2cm in 54.5% of the cases, with lymph node involvement in 45.5% (Table 1).

Eleven patients were in menopause and none of them used hormonal replacement therapy. Eleven patients reported familial history of cancer, including breast cancer, esophageal, cervical and ovarian cancer. Fifteen patients were Luminal, while six were Triple Negative and only one was HER2-enriched.

All patients underwent to surgery, but four patients with neoadjuvant chemotherapy and 16 patients with adjuvant therapy.

3.1 *NOS3* and *TCF7L2* variant analysis

The *NOS3* c.-813C>T showed significance for allele C related to history of familial cancer (p=0.022) (Table 2). The c.896G variant pointed to happen in obesity in the Luminal group (p=0.0849) (Table 3). Intron 4 VNTR b/a showed no statistical relevance in any parameter analyzed. Characteristics like abortion, contraceptive, menopausal, and breast cancer subtype did not show any statistical significance for the variants evaluated. Regarding TCF7L2 variants no statistical relevance was observed for the parameter analyzed.

3.2 *NOS3* and *TCF7L2* expression

Despite *NOS3* expression could not be related to any parameter, it sugegested a relation in group of patients with systemic arterial hypertension (SAH) (p=0.0795).

According to BMI grade, *TCF7L2* expression showed significance for overweight compared to eutrophic patients (p=0.0478), but no significance was found for obese group (Figure 1). No correlation was found between the presence of the variant and the expression level of *NOS3* or *TCF7L2* genes.

4 Discussion

It was observed an average age of breast cancer very similar to Mexican and Indian population [4,11]. *NOS3* variants have been studied in many cancers, including breast cancer, not only as biomarker for developing and progression [5,12–14], but also as predicting treatment efficacy [15]. Besides that, c.-813C and c.894T alleles seem to be associated with breast cancer recurrence and death, in Korean women with ER positive tumors [16].

The *NOS3* c.-813C>T variant was never associated to familial breast cancer risk, like BRCA and CDH1 genes [17].

The possible correlation *NOS3* expression is known to be related to tumor growth and regulation of Notch pathway [18]. In our analysis, the expression of *NOS3* showed a tendency for SAH, but no variant could be related, as observed in non-cancer Indian patients that showed association with VNTR 4a allele [19]. *NOS3* c.894T>G variant in our study for obesity in the Luminal group could be a highlight to understand a previous report about the contribution of obesity to an increased risk of postmenopausal luminal disease, but not to risk of either triple negative or HER2-enriched breast cancer subtypes [20]. For Intron 4 VNTR (4a/b) variant, the genotypes a/a and a/b were reported to be associated with breast cancer in Mexican women [4], but no association was found in our group of patients.

In the current climate of food overabundance and sedentary lifestyle, the genetic background could lead to metabolically disadvantageous phenotypes, inducing many diseases. The genetic architecture of obesity has been evaluated [21] and genes associated to Wnt pathway may play a role in obesity and also in cancer [22,23]. Wnt signaling increases cytosolic levels of β-catenin, its translocation into the nucleus to bind TCF7L2 and enhance the expression of target genes like CCND1 and c-MYC oncogenes [24]. In German patients, T allele of g.103894G>T variant showed an association with significance with breast cancer risk, suggesting a possible influence on the risk of familial BC [25]. In Northwest Chinese women *TCF7L2* variants g.53341C>T was not associated to breast cancer risk [26], in agreement with our results. In our group, variants *TCF7L2* showed no influence in breast cancer according to the parameters analyzed.

NOS3 expression is known to be related to tumor growth and regulation of Notch pathway [18]. In our analysis, the expression of *NOS3* showed a tendency for SAH, but no variant could be related, as observed in non-cancer Indian patients that showed association with VNTR 4a allele [19].

TCF7L2 expression was already correlated with esophageal cancer, in japanese patients using immunohistochemical analysis, with a poor prognosis [27]. It was already related to physiological response in patients underwent bariatric surgery [28]. No previous report was found demonstrating a relation between *TCF7L2* expression and overweight in breast cancer patients.

In summary, *NOS3* c.-813C>T showed significance for history of familial cancer and *TCF7L2* expression as significance for overweight patients. Despeite the limitations of this study like small number of patients enrolled and also absence of control group for determining the profile of *NOS3* and *TCF7L2* expression. Since *TCF7L2* still is under investigation in litrature, we conclude that eNOS is a best candidate for future medical interventions, to have a better response in cancer treatment, since its impact in cancer have already been proved. Therefore, further studies with large cohort applied to case-control groups could determine the real role of this two genes in breast cancer.

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Figure legends

Figure 1. TCF7L2 mRNA levels by patient weight.

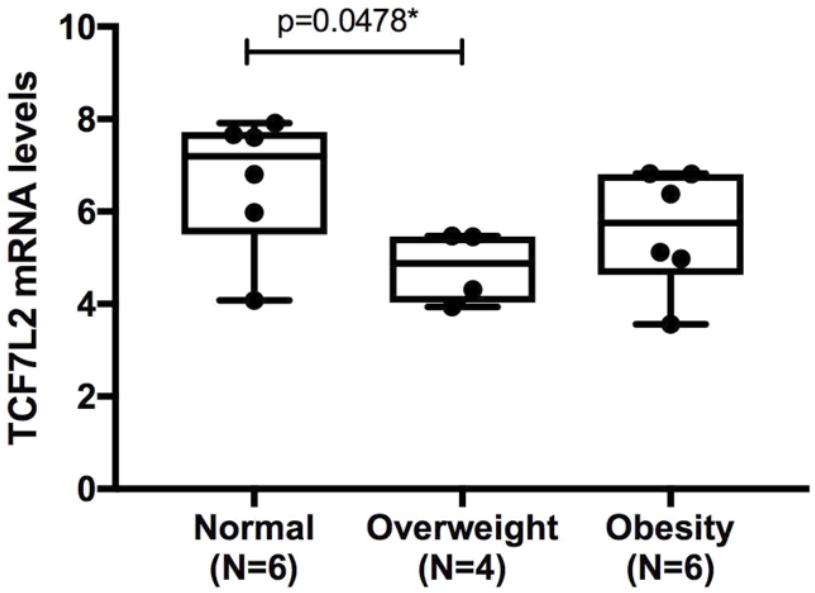
p=0.0478*
TCF7L2 mRNA levels
10
8
6
4
2
0
Normal
(N=6)
Overweight
(N=4)
Obesity
(N=6)

Table legends

Table 1. Patients data releted do gene expression.
Table 2. CA History in 2 groups (comparing with genotype / eNOS allele).
Table 3. Obesity in 2 groups (comparing with genotype / eNOS allele).

Characteristic	Patient (n= 22)			
	N	%	p value NOS3	p value TCF7L2
Age			0.9853	0.3127
≤ 50	5	22.7		
>50	17	77.3		
Skin Color			0.2462	0.1555
White	3	13.6		
Black	3	13.6		
Brown	16	72.8		
Menopause				
Yes	9	40.9	0.8588	0.3154
No	10	45.5		
Unknown	3	13.6		
Tumor size			0.8182	0.2330
≤ 2cm	4	18.2		
≥2cm	12	54.5		
Unknown	6	27.3		
Committed Lymp Nodes				
Yes	10	45.4	0.8588	0.6038
No	9	41.0		
Unknown	3	13.6		
Staging (TNM)				
I e II	12	54.5	0.5152	0.6511
III	9	41.0		
Unknown	1	4.5		
History Family Cancer				
Yes	11	45.4	0.7640	0.4967
No	9	41.0		
Unknown	3	13.6		

	SNPs	Genotype allele	All patientsn=14 (%)	CA Hist. No n= 8 (%)	CA Hist. Yes n= 6 (%)	P value	OR (95% CI)
eNOS	**T-786C**	T/T	9 (64.29)	7 (87.50)	2 (50.00)	Ref	
		T/C	5 (35.71)	1 (12.50)	4 (50.00)	0.09	14.00 (0.94-207.7)
		T	75.00	93.75	50.00	Ref	
		C	25.00	6.25	50.00	0.02	14.00 (1.37-143.0)
	G894T	G/G	10 (71.43)	6 (75.00)	4 (66.67)	Ref	
		G/T	4 (28.57)	2 (25.00)	2 (33.33)	1.00	1.50 (0.15-15.47)
		G	85.71	87.50	83.33	Ref	
		T	14.29	12.50	16.67	1.00	1.40 (0.17-11.69)
	VNTR 4b/a	b/b	7 (50.00)	4 (50.00)	3 (50.00)	Ref	
		a/b	7 (50.00)	4 (50.00)	3 (50.00)	1.00	1.00 (0.12-8.31)
		b	75.00	75.00	75.00	Ref	
		a	25.00	25.00	25.00	1.00	1.00 (0.18-5.64)

	SNPs	Genotype allele	All patients n=14 (%)	Non-Obese n= 8 (%)	Obese n= 6 (%)	P value	OR (95% CI)
eNOS	**T-786C**	T/T	8 (57.14)	4 (50.00)	4 (66.67)	Ref	
		T/C*	6* (42.86)	4* (50.00)	2* (33.33)	0.63	0.50 (0.06-4.48)
		T	71.43	68.75	75.00	Ref	
		C	28.57	31.25	25.00	1.00	0.73 (0.14-3.94)
	G894T	G/G	10 (71.43)	4 (50.00)	6 (100.0)	Ref	
		G/T	4 (28.57)	4 (50.00)	0 (0.00)	0.08	0.08 (0.01-1.81)
		G	85.71	75.00	100.0	Ref	
		T	14.29	25.00	0.00	0.11	0.11 (0.01-2.29)
	VNTR 4b/a	b/b	6 (42.86)	4 (50.00)	2 (33.33)	Ref	
		a/b	8 (57.14)	4 (50.00)	4 (66.66)	0.63	2.00 (0.22-17.90)
		b	71.43	75.00	66.67	Ref	
		a	28.57	25.00	33.33	0.69	1.50 (0.29-7.81)

4 CONCLUSÃO

Tendo em vista o impacto mundial dos cânceres cervical e de mama na saúde da mulher, é relevante que se analisem constantemente marcadores que auxiliem no diagnóstico precoce e/ou controle das doenças. O acesso a novos biomarcadores, independente da fase da doença, já mudou para melhor a qualidade de vida de muitos pacientes. Assim, promover o estudo de marcadores como DEK, NOS3 e TCF7L2, pode levar à melhoria dos painéis de prognóstico e até a um controle mais eficaz de neoplasias.

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APÊNDICE A – THE UNIQUE DEK ONCOPROTEIN IN WOMEN'S HEALTH: A POTENTIAL NOVEL BIOMARKER

Ana Cecília de Albuquerque Oliveira 1, Ferdinand Kappes 2, Danyelly Bruneska Gondim Martins 1,3,*, José Luiz de Lima Filho 1,3

1 - Molecular Prospecting and Bioinformatics Group – Laboratory of Immunopathology Keizo Asami (LIKA) – Federal University of Pernambuco (UFPE). Av. Prof. Moraes Rego, 1235 - Cidade Universitária, Recife - PE, Postal Code 50670-901, Brazil.

2 - Department of Biological Sciences, Xi'an Jiaotong-Liverpool University No 111, Ren Ai Road, Dushu Lake Higher Education Town, Suzhou Industrial Park (SIP), Suzhou, 215123, P.R. China.

3 - Department of Biochemistry – Federal University of Pernambuco (UFPE), Brazil. Av. Prof. Moraes Rego, 1235 - Cidade Universitária, Recife - PE, Postal Code 50670-901, Brazil

*Corresponding author:

bruneska@prospecmol.org

Av. Prof. Moraes Rego, 1235 – Cidade Universitária, Recife - PE, Brazil, CEP: 50670-901. Phone: +55 81 2126-8484

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Abstract

Breast and cervical cancer are the first and fourth cancer types with the highest prevalence in women, respectively. The developmental profiles of cancer in women can vary by genetic markers and cellular events, and, in turn, are strongly influenced by genetics, age, and lifestyle. Due to the dynamic and reversible nature epigenetic mechanisms have been proposed as targets for a series of treatment approaches, especially in tumor therapy. The human DEK protein, a histone chaperone, belongs to a specific subclass of chromatin topology modulators, being involved in the regulation of DNA-dependent processes. The expression patterns of DEK vary between healthy and cancer cells. High expression of DEK is associated with poor prognosis in many cancer types, suggesting that DEK takes part in oncogenic activities via different molecular pathways, including inhibition of senescence and apoptosis. The focus of this review was to highlight the molecular role of the DEK protein in these two female cancers.

Keywords: DEK oncogene, human papillomavirus, breast carcinoma, cervical neoplasm.

1. Introduction

The developmental profiles of cancer in women can be highly variable as revealed by genetic markers. Age and lifestyle strongly influence cellular and epigenetic events [1,2]. A series of genetic alterations accumulates over time and induces changes in molecular pathways which accelerate the initiation and clonal dominance of mutations in the stem and progenitor cells [3]. Age-standardized analysis showed that the incidence of breast cancer increases with age, whereas the frequency of cervical cancer peaks at age 55, and it is found to decline with further aging [3,4].

Cellular performance is under constant regulation triggered by intra- as well as the extracellular environment, that together, amongst other vital regulatory circuits, trigger epigenetic events resulting from various interactions. Epigenetics refers to largely non-mutational regulatory events in the genome that alter gene expression resulting typically in activation of oncogenes [5]. These epigenetic changes include alterations in DNA methylation, histone post-translational modifications [6], unbalanced recruitment of chromatin remodeling factors, and altered expression of micro RNAs (miRNAs) and long non-coding RNA (lncRNAs) [7]. Tumor development in a wide range of sporadic cancers is implicated with abnormal methylation leading to silencing of tumor suppressor genes [8].

Epigenetic mechanisms are of dynamic and reversible nature and are being proposed as targets for treatment especially in cancer [9]. Even though DNA methylation has been discovered first, histone post-translational modifications and their specific misregulation in cancer have risen to prominence in cancer research [10]. The DEK molecule, encoded by the proto-oncogene DEK [11], has gained visibility in this field as its expression patterns and molecular functions are intimately tied to cancer progression. In particular epigenetic events regulating cell cycle and survival functions have been associated with this unique, highly conserved, and biochemically distinct nuclear factor [12,13].

DEK is referred as “gatekeeper” of chromatin, as it controls chromatin integrity by restricting broad access to histones by dedicated chaperones [14], and exhibits a series of biochemical functions, such as alterations to the superhelical density of DNA in chromatin [15]. These functions are potentially involved in the regulation of important pathways like p53, Wnt/β-catenin, mTOR, Rho and NF-K β signaling and Vascular Endothelial Growth Factor (VEGF) [13,16–18]. Therefore, deregulation of DEK could be involved in cancer development and maintenance. In fact, DEK overexpression has been related to poor prognosis in many types of cancer like melanoma [19], hepatocellular carcinoma [20,21], bladder cancer [22], brain tumor [23], gastric adenocarcinoma [24], coloretal cancer [25], breast cancer [26,27], and also others [28–31]. Overexpression of DEK was correlated with poor survival in solid tumors, which suggests that the expression status of DEK is a valuable biomarker for the prediction of prognosis and serves as a novel therapeutic target [32].

Breast and cervical cancer are among the cancer types with the highest prevalence in women. Breast cancer is the most frequent cancer among women, being the second most common cancer worldwide and ranks fifth as the cause of death from cancer [33]. Common risk factors for the development of breast cancer are reproductive status, family history, obesity, and lifestyle, which include alcoholism and smoking habits [34]. Compounds found in food and drinking water may also increase breast cancer risk once they can act as endocrine disruptors leading to altered epigenetic hormonal regulation [7]. Even though genetic mutations in BRCA gene are well known to contribute to heritage cancer, 90% of breast cancers are sporadic and the molecular roles of specific genes in the development of different human breast cancer subtypes remaining only partially understood [35].

Human Papillomavirus (HPV) is the leading cause of cervical cancer which represents the fourth most common cancer, accounting for 7.5% of all fatalities in women [33]. The early onset of unprotected sexual activity with multiple sexual partners increases the risk of HPV infection. Additionally, the use of oral contraceptives, socioeconomic and smoking status may increase the risk of viral infection. In most cases, the viral load remains subclinical and is cleared by the immune system [36]. Current epidemiological and molecular studies have shown that HPV infection, along with genetic and epigenetic changes, are associated and essential for initiation, development, and progression of cervical cancer [6].

The aim of this paper was to review the discovery of DEK and its molecular functions in breast and cervical cancer, highlighting its potential as a novel biomarker and target for cancer treatment in women health.

2. Epigenetics and DEK

Epigenetics refers to the study of potentially heritable changes in gene expression patterns that occur without a change in the DNA sequence. Many studies have shown that epigenetic mechanisms provide an “extra” layer of transcriptional control that regulates how genes are expressed [37]. The factors that control chromatin biology can be grouped into four broad classes: histone modifiers, chromatin remodelers, histone variants, and histone chaperones [38].

Histone chaperone complexes play important roles in the regulation of telomeres, gene transcription and heterochromatin, alterations of which ultimately contribute to disease pathogenesis [39]. These chaperones are able to associate with histones upon their synthesis, escort them into the nucleus, and aid in their specific association with DNA during different processes such as DNA replication, repair, or transcription [38].

In cellular chromatin structure, DEK exhibits functions typically observed in factors known as Suppressors of Variegation Su(var) - nuclear proteins which positively regulate pericentromeric heterochromatin in the interphase nucleus. They are essential for maintaining a proper cell-type-specific balance between euchromatin and heterochromatin

[40]. DEK serves as histone chaperone via phosphorylation by forming complexes with CK2 [41]. Furthermore, it is necessary for the maintenance of heterochromatin integrity, and facilitates the interaction between Heterochromatin Protein 1a (HP-1) and trimethylated histone H3 at lysine 9 (H3K9me3) [42].

In mammalian cells, the assembly of histone variant H3.3, along with H4, into nucleosomes via the replication-independent pathway is mediated by multiple histone chaperones, including DEK [39]. Immunoprecipitation studies have confirmed that DEK associates with activating histone modifications such as H3K4me2/3 in addition to repressive modifications, such as H3K9me3 [41]. After interacting with histones, DEK can exert a potent inhibitory effect on both p300 and its paralog CREB-binding protein (CBP), forming the p300/CBP-associated factor (PCAF)-mediated histone acetyltransferase activity, which can also alter gene transcription [43].

The interaction of DEK with DNA is rather structure-specific than sequence-specific with a strong preference for distorted DNA structures, such as supercoiled or cruciform DNA forms [44]. The first pro-oncogenic function for DEK was proposed in virtue of its initial cloning as the chimeric transcript of DEK and CAN/NUP214, caused by the chromosomal translocation t(6;9)(p23;q34) identified in 1% of acute myelogenous leukemia patients (AML) [11,45].

Immediately after its initial discovery, DEK was associated with functions in autoimmune disorders. Circulating autoantibodies against DEK were found in the blood of patients with autoimmune diseases such as juvenile rheumatoid arthritis [46], systemic lupus erythematous, and sarcoidosis [47–50]. Interestingly, DEK was identified as a critical protein in molecular events during implantation in the endometrium of mice in early pregnancy, by influencing stromal cell decidualization. These studies shed light on potential new functions for DEK in women health, as such events are related to DNA repair, cell proliferation, and apoptosis [51].

3. DEK structure

The DEK gene is located on chromosome 6p22.3 [11] with the resulting protein comprising 375 amino acids and belonging to a specific subclass of chromatin topology modulators (Fig.1a). DEK is typically found associated with chromatin in the nucleus under steady-state conditions [52], influencing the availability of DNA for transcription. It has been classified as a general chromatin structural protein due to its ability to change the topology of DNA in chromatin [15].

DEK comprises a Scaffold Attachment Factor (SAF) motif, also found in a variety of other nuclear proteins involved in transcription, DNA repair, RNA processing, and apoptotic chromatin degradation [53,54]. With this 35 amino acid residues long stretch DEK primarily interacts with DNA. The scaffold attachment factors A and B (SAF-A and -B) are nuclear proteins that bind to AT-rich chromosomal regions

known as Scaffold- or Matrix-Attachment Regions (SAR/MAR). Interestingly, significant similarities of this domain are observed in several other chromatin-associated proteins, such as the N-terminal region of human S, a caspase-3-activated protein required for apoptotic chromatin condensation, and the PIAS (protein inhibitor of the activated signal transducer and activator of transcription, STAT) [55]. Therefore, this motif was additionally named SAP after identification the three members: SAF-A/B, Acinus and PIAS [56]. The SAP-containing factors are predominantly found in the N-terminal region. However, in DEK the SAP-box is located centrally between residues 149 to 187, and is accessorized with a pseudo-SAP motif located at the N-terminal of the SAP-box, which distinguishes DEK from all other SAP-motif-containing factors (Fig.1b) [54,56].

The pseudo-SAF/SAP-motif region appears to be the dominant DNA-interacting and modulating hub, responsible for most of DEK's DNA-related functions [53]. This function can be exemplified by the generation of large nucleo-protein complexes via inter- and intramolecular interactions of this domain with DNA [44]. A second DNA-interacting domain at the C-terminal portion of DEK (Fig.1a, residues 270-350) is involved in the regulation of the overall DNA binding activity, which is controlled through phosphorylation by the protein kinase CK2 and results in the stimulation of DEK molecule multimerization. Even though CK2 appears to be the dominant kinase regulating DEK functions, a series of phosphorylation sites have been mapped, which are set by other kinases [53,57,58].

Detailed knowledge about the impact of the various phosphorylation events and other post-translational modifications remain mostly obscure at the current time, however they are believed to play essential role in tumor development.

4. DEK Regulation

Given its involvement in global and local chromatin structure, it is not surprising that multiple studies have shown that DEK functions are involved in the regulation of transcription, mRNA splicing, DNA damage repair and cell cycle control [15,59–61]. To highlight the importance of DEK in maintaining global chromatin integrity *in vivo* is essential to observe the intense regulation based on three known post-translational mechanisms relevant to DEK: phosphorylation, acetylation, and covalent or non-covalent poly-ADP-ribosylation (Fig.1a).

The most important regulatory mechanism occurs through CK2 phosphorylation events on the C-terminal domain of DEK, which enhances its multimerization and regulates its DNA-binding activities. The phosphorylation events weaken the binding of DEK to DNA, although cellular analysis reveal that phosphorylated DEK remains attached to chromatin. Unphosphorylated or underphosphorylated forms of DEK remain connected to chromatin throughout the cell cycle, but the phosphorylation of DEK in late G1 phase triggered by CK2 modifies the interaction of DEK with DNA and chromatin,

resulting in less stable binding. Altogether, more than 42 phosphorylation sites have been mapped in DEK, suggesting the presence of complex regulatory circuits that integrate and regulate its bona fide, disease, and stress-related functions [53,57].

The second mechanism of regulation is based on the formation of the complex PCAF that regulates DEK functions through acetylation, and decreases its affinity to DNA in general and within promoter regions [62]. High concentrations of PCAF drive DEK into Interchromatin Granule Clusters (IGCs), which can be blocked using a synthetic cell permeable PCAF inhibitor [63].

Under stress conditions, a different regulation occurs through covalent (but also non-covalent) modifications set by poly(ADP-ribose) polymerase-1 (PARP1, ARTD1), a molecular DNA damage sensor that catalyzes the synthesis of the compound biopolymer poly(ADP-ribose) (PAR) under consumption of NAD+. PARylation leads to the removal of DEK from chromatin to allow the access of the transcription machinery, or DNA replication or repair complexes [64]. In the HeLa cell system, assembly of the Preinitiation Complex (PIC) depends on the presence of the chaperone SET or the activity of PARP and requires the exclusion of DEK (and perhaps PARP1) from chromatin. Removal of DEK rendered chromatin accessible to endonuclease digestion but did not permit mediator recruitment or transcription, in the absence of SET [64].

As example of DEK repression, miR-592 can target the 3'-UTR region of the DEK mRNA, repressing its translation and suppressing cell growth in hepatocellular carcinoma (HCC) [65]. DEK is also suggested to be a direct target of miR-489, which is highly expressed in quiescent satellite cells and downregulated during satellite cell activation [66]. Thus, miR-592 and miR-489 could suppress DEK, which reinforces its potential as therapeutic target in cancer.

5. The role of DEK in cancer

The expression patterns of DEK are substantially different in healthy and cancer cells. High expression of DEK is associated with poor prognosis in many cancer types. It suggests that DEK takes part in oncogenic activities through different molecular pathways, including involvement in initial steps of developing oncogenic mutations that lead to cancer initiation [67,68], inhibition of senescence and apoptosis [16], promotion of cell growth and mobility [69], and enhancement of tumor growth and metastasis [12,19]. A general overview of the most significant findings relating to DEK function is given in Fig.2.

5.1 DEK and Cervical Cancer

In healthy cells, DEK is under the tight regulation and direct control of E2F transcription factors, but it is repressed by p16 and pRB. Loss of pRB function is believed to lead to inappropriate activation of E2F, together with the activation of the p53 tumor

suppressor. Then, the combined suppression of pRB and p53 pathways, caused by HPV proteins, is critical for disabling the cellular defense against neoplasia, allowing increased cellular proliferation rates and cervical tumorigeneses [28,67].

HPV is the leading cause of cervical cancer, and HPV infection is also related to other neoplasms such as cancer of the vagina, vulva, head and neck, anal, and even penile carcinomas [36]. Most of the HPV genotypes cause no clinical symptoms and are controlled by the immune system, with clearance typically occurring within one or two years. However, cervical lesions that progress to cancer are associated to an oncogenic subset of HPVs classified as high-risk HPV types [70].

HPV infects both cutaneous and mucosal squamous epithelium, with an exclusive intra-epithelial cycle. The infection with high-risk HPV triggers the four most significant steps in cervical cancer development: infection of metaplastic epithelium at the cervical transformation zone; viral persistence; progression of the persistently infected epithelium to cervical pre-cancer; and invasion through the basement membrane of the epithelium [4].

The high-risk HPV genotypes are responsible for the genetic instability of the infected cells, caused by its oncogenes E6 and E7 that were implicated in cell cycle deregulation through p53 and pRB inactivation and degradation [71]. The viral genome can be integrated into genome host, losing the expression of the regulatory E2 protein and allowing E6 and E7 viral proteins to act in an uncontrolled mode (Fig.3a).

In normal keratinocytes, DEK overexpression was sufficient for causing oncogenic phenotypes, while loss of DEK in vitro inhibited cervical cancer cell proliferation, migration and invasion [72,73]. HPV E7 indirectly induces expression of the human DEK gene, both *in vitro* and *in vivo*. Conversely, DEK loss results in cell death in HPV-positive cervical cancer cells, in part through p53 activation, while DEK knockout mice are relatively resistant to the development of chemically induced skin papillomas [16]. This scenario shows that DEK overexpression in primary cells represents an efficient bypass of the senescence process towards immortality, but also suggests that HPV oncoproteins are involved in the upregulation of DEK [73]. Therefore, DEK plays an essential role in the early stage of cervical carcinogenesis, and can be helpful for early diagnosis and also a potential therapeutic target for cervical cancer, collaborating with tools for disease control in women [74].

5.2 DEK and Breast Cancer

Breast cancer is a complex and heterogeneous disease with three main subtypes: Estrogen Receptor (ER) positive breast cancer, Human Epidermal Growth Factor Receptor 2 (HER2) overexpressing breast cancer, and Triple-Negative Breast Cancer (TNBC) or basal-like breast cancer [75].

An involvement of DEK in breast cancer is observed by the up-regulation of transcripts

in lymph nodes of human breast tumors and is generally related to poor prognosis [27]. Subsequent studies showed strong association of DEK to breast cancer cell lines from high-grade primary invasive ductal carcinomas, yet not to benign tissue and low-grade carcinomas [69]. In mammosphere cultures, DEK overexpression has leveraged the population size and mammosphere formation, when compared with a population with depleted DEK cells [69]. Furthermore, DEK has been related to metastasis in lung cancer as seen in cDNA studies, which is the same for cell motility and invasive potential in both healthy and breast cancer cells [69,76].

DEK is associated with hormone-stimulated proliferation in the ER+ since it is an estrogen receptor target gene up-regulated by estrogen exposure and down-regulated by tamoxifen-mediated therapy (Fig.3b). In breast cancer prognosis, the estrogen receptor-positive status (ER+) cell presents a better response to treatment for the standard therapy with tamoxifen, an estrogen receptor antagonist. Despite the fact that DEK protein levels decrease by tamoxifen treatment, these levels are still detectable. Therefore, the influence of DEK was tested in a model of DEK depletion by short hairpin RNA (shRNA), showing increased apoptotic response to tamoxifen [42]. It indicates that DEK depletion and tamoxifen treatment could act against ER^+ cancer cells.

DEK also was shown to contribute to tumor growth and metastasis stabilizing a downstream relation with Recepteur d'Origine Nantais (RON) which belongs to the Receptor Tyrosine Kinases (RTK) subfamily [17]. These contributions are presumably correlated with Wnt expression and subsequent β-catenin activity in human breast cancer. The importance of DEK in tumor angiogenesis was pointed out as a key regulator of Vascular Endothelial Growth Factor (VEGF). In the nucleus, DEK is recruited to regions with DEK-response element (DRE) and Hypoxia Response Element (HRE) in the VEGF promoter and enhances the recruitment of Hypoxia-inducible factor 1-alpha (HIF-1α) and p300. These conditions are sufficient to increase vascular proliferation in endothelial cells, migration, and cell growth [18]. In TNBC cells, DEK promotes proliferation, angiongenesis and metastasis via PI3K/AKT/mTOR signaling pathway, being pointed as a potential target in TNBC therapy [77].

6. DEK as therapeutic approach

The DEK molecule is currently used as a urinary marker in the development of novel approaches in advanced marker for bladder cancer [78]. It is also suggested as a complete pathological response to treatment in locally advanced rectal cancer [79]. Depletion of DEK in tumor cell cultures results in sensitivity to genotoxic agents, particularly in proliferating cells, suggesting that DEK overexpression may be correlated with adverse clinical response to clastogenic therapies [80]. In colorectal cancers, this condition was positively correlated with tumor size, grade, lymph node metastasis, serosal invasion, late stage, disease-free survival and 5-year survival rates. Moreover, patients with late-stage

colorectal cancer and high DEK expression have had worse survival rates than those with low DEK expression [81].

Aligned with new therapeutic approaches, a DEK-targeted aptamer was evaluated against joint inflammation in vivo, showing impairment in the ability of neutrophils to form neutrophil extracellular traps (NETs) and significant reduction of the inflammatory process [82].

Since DEK depletion is related to proliferation, migration, and invasion [72]; and it has enhanced the apoptotic response to tamoxifen in ER^+ breast cancer cells [42], specific particles like aptamers could also be developed to control DEK expression in cancers like cervical cancer. These findings reinforce the potential of DEK as biomarker for breast and cervical cancer, although many questions remain to be elucidated about the involvement of DEK in these cancers.

7. Conclusions

The incidence of breast and cervical cancer are, together, a major cause of women death around the world. This review has focused on the role of the unique DEK protein in nuclear processes in the background of breast and cervical cancer development and prognosis. The available data strongly suggests that targeting DEK functions represents a strategy that will expand routes for treatments that benefit women health. Therefore, further research is required to definitively establish this molecule in the genetic panels for cancer prognostic tool and as a potential therapeutic target in these cancers.

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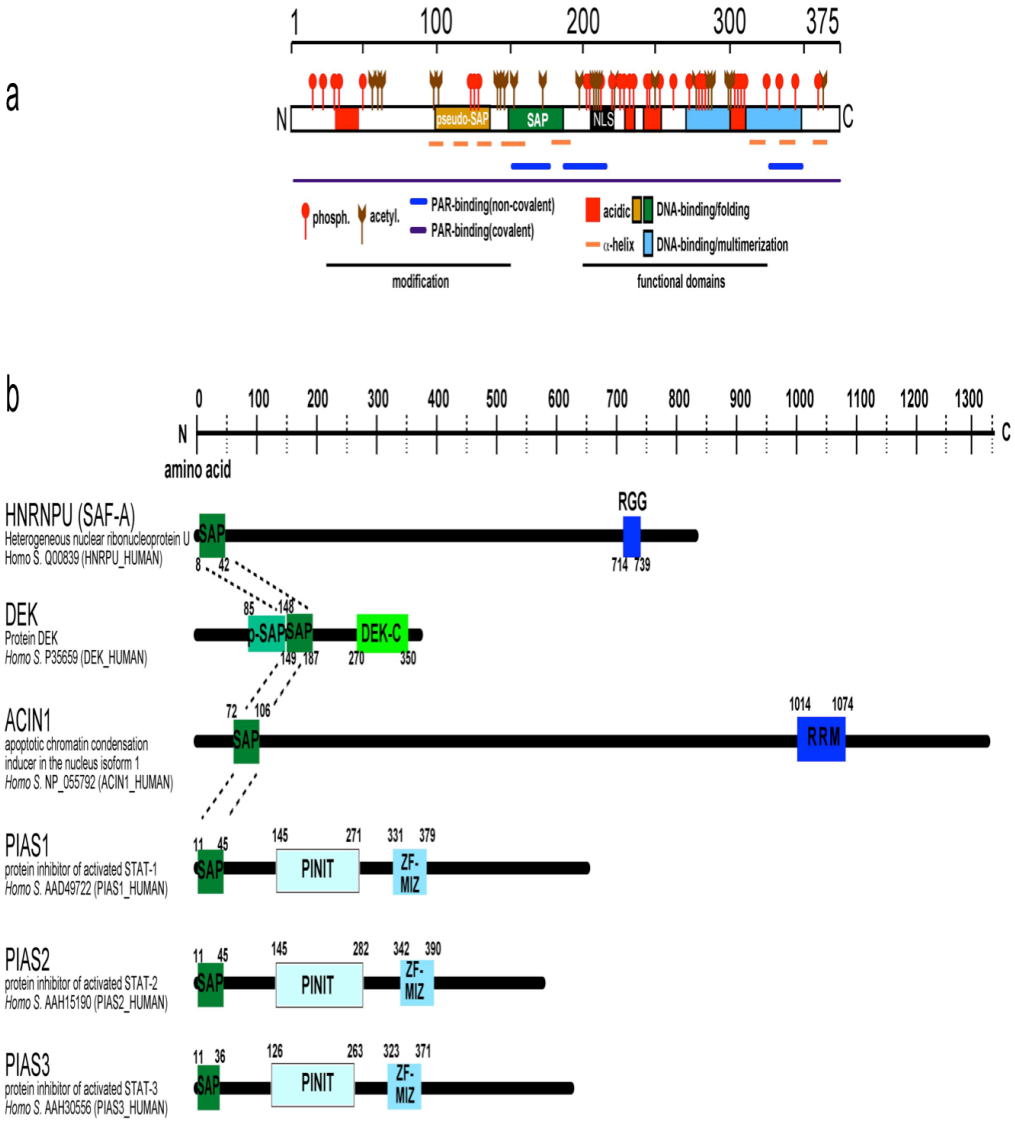
Figure Legends

Fig.1. DEK structure and regions. (a) Schematic structure of the human DEK oncogene, highlighting important residues for post-translational modifications by phosphorilation, acetylation and PAR-binding; and functional domains for DNA-binding. (b) Alignment of SAP/SAF domains from different proteins. SAP domains (dark green) of indicated proteins were aligned, showing the unusual position of the unique pseudo-SAP/SAP domain in DEK. Particular other characteristics in the chosen proteins are shown. In the HNRNPU protein, the C-terminal domain RGG (dark blue) is responsible for its RNA binding activity [83]. The DEK-C (light green) domain has been related to reversion of abnormal DNA-mutagen sensitivity. The ACIN1 protein has a RRM domain (dark blue), a RNA recognition motif that is a conserved domain among many proteins, such as those involved in RNA regulation and stability [84]. The family of PIAS proteins have two characteristic domains PINIT (light blue), that plays a role in nuclear retention of PIAS3 [85], and a zinc finger domain ZF-MIZ (cyan blue).

Fig.2. Milestones in DEK research that have paved the way for scientific and clinical cancer findings relating to DEK functions in cancer.

Fig.3. (a) In normal cells, DEK promoter is under the direct control of pRB function as it controls E2F transcription factors. Loss of pRB function, caused by HPV E7 protein, leads to inappropriate release of E2F factor. It activates DEK overexpression, resulting in increased cellular proliferation rates and tumourigenesis. (b) DEK is observed to be up-regulated in breast cancer. DEK gene is an estrogen receptor target up-regulated by estrogen exposure and downregulated by tamoxifen-mediated therapy. In breast cancer cells, DEK is recruited to regions with DEK response element (DRE) and Hypoxia Response Element (HRE) in the VEGF promoter, enhancing the recruitment of Hypoxia-inducible factor 1-alpha (HIF-1α) and p300, contributing to the tumorigenesis.


a
1
100
200
300
375
N
C
pseudo-SAP
SAP
NLS
phosph.
acetyl.
PAR-binding(non-covalent)
PAR-binding(covalent)
acidic
DNA-binding/folding
α-helix
DNA-binding/multimerization
modification
functional domains
b
0
100
200
300
400
500
600
700
800
900
1000
1100
1200
1300
N
C
amino acid
HNRNPU (SAF-A)
Heterogeneous nuclear ribonucleoprotein U
Homo S. Q00839 (HNRPU_HUMAN)
SAP
RGG
8
42
714
739
DEK
Protein DEK
Homo S. P35659 (DEK_HUMAN)
85
148
SAP
SAP
DEK-C
149
187
270
350
ACIN1
apoptotic chromatin condensation
inducer in the nucleus isoform 1
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72
106
SAP
1014
1074
RRM
PIAS1
protein inhibitor of activated STAT-1
Homo S. AAD49722 (PIAS1_HUMAN)
11
45
SAP
145
271
PINIT
331
379
ZF-MIZ
PIAS2
protein inhibitor of activated STAT-2
Homo S. AAH15190 (PIAS2_HUMAN)
11
45
SAP
145
282
PINIT
342
390
ZF-MIZ
PIAS3
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36
SAP
126
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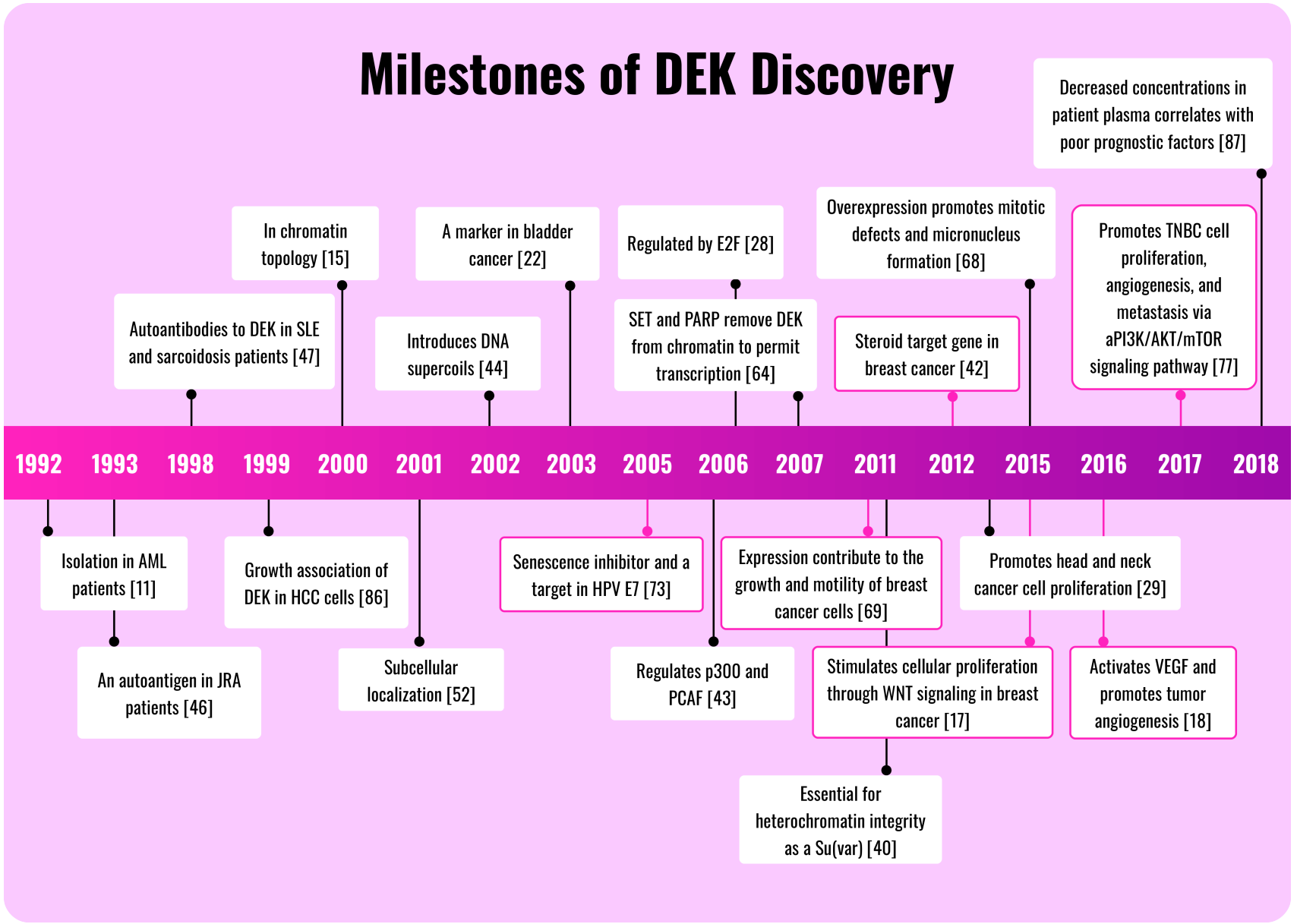
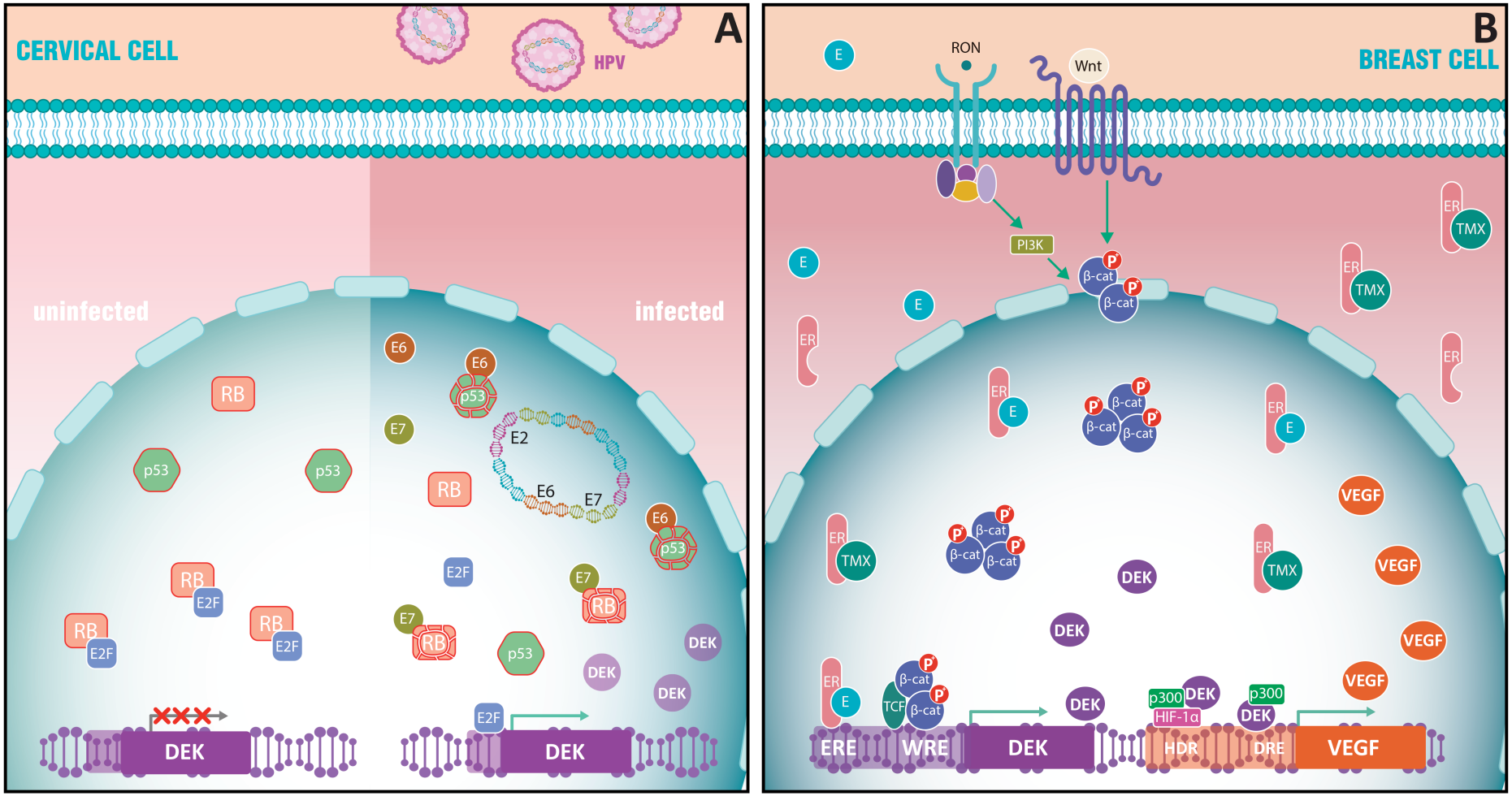
PINIT
323
371
ZF-MIZ


Milestones of DEK Discovery
1992
Isolation in AML patients [11]
1993
An autoantigen in JRA patients [46]
1998
Autoantibodies to DEK in SLE and sarcoidosis patients [47]
1999
Growth association of DEK in HCC cells [86]
2000
In chromatin topology [15]
2001
Subcellular localization [52]
2002
Introduces DNA supercoils [44]
2003
A marker in bladder cancer [22]
2005
Senescence inhibitor and a target in HPV E7 [73]
2006
Regulates p300 and PCAF [43]
Regulated by E2F [28]
SET and PARP remove DEK from chromatin to permit transcription [64]
2007
2011
Expression contribute to the growth and motility of breast cancer cells [69]
Stimulates cellular proliferation through WNT signaling in breast cancer [17]
Essential for heterochromatin integrity as a Su(var) [40]
2012
Steroid target gene in breast cancer [42]
2015
Overexpression promotes mitotic defects and micronucleus formation [68]
Promotes head and neck cancer cell proliferation [29]
2016
Activates VEGF and promotes tumor angiogenesis [18]
2017
Promotes TNBC cell proliferation, angiogenesis, and metastasis via aPI3K/AKT/mTOR signaling pathway [77]
2018
Decreased concentrations in patient plasma correlates with poor prognostic factors [87]


A
CERVICAL CELL
HPV
uninfected
infected
RB
p53
E6
E7
E2
E2F
DEK
B
BREAST CELL
RON
Wnt
PI3K
β-cat
ER
TMX
E
VEGF
TCF
p300
HIF-1α
ERE
WRE
HDR
DRE