Universidade Federal do Rio Grande do Sul
Faculdade de Medicina
Programa de Pós-Graduação em Medicina: Ciências Médicas
Mestrado e Doutorado

Prevalência da mutação de gene *ESR1* em pacientes com câncer de mama receptor hormonal positivo resistentes à terapia endócrina neoadjuvante

TESE DE DOUTORADO

Tomás Reinert

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Orientadora: Profa. Dra. Marcia Silveira Graudenz

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"Nothing in biology makes sense except in the light of evolution."

— **Theodosius Dobzhansky**

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RESUMO

Introdução: Mutações no gene *ESR1* (ESR1m) são importante mecanismo de resistência à terapia endócrina em câncer de mama receptor de estrogênio positivo (RE+) e vêm sido reconhecidas como biomarcadores prognósticos e preditivos, assim como um potencial alvo terapêutico. Entretanto, as pesquisas publicadas descrevem ESR1m como um potencial mecanismo de resistência adquirida no contexto de doença metastática. O papel de *ESR1*m como potencial mecanismo de resistência intrínseca no contexto de doença inicial não foi estudada. O escore PEPI é um índice que avalia o perfil de sensibilidade ou resistência à terapia endócrina neoadjuvante. O objetivo deste estudo foi avaliar a prevalência de ESR1m em amostras tumorais de pacientes com câncer de mama RE+ com perfil de resistência à terapia endócrina neoadjuvante com inibidores de aromatase.

Resultados: Uma coorte prospectiva de mulheres na pós-menopausa com câncer de mama RE+ HER2-negativo estádio II-III tratadas com hormonioterapia neoadjuvante foi conduzida. Pacientes receberam tratamento com anastrozol por um período mínimo de três meses. Amostras tumorais de pacientes com um perfil de resistência endócrina primária (definida como escore PEPI maior ou igual a 4) foram selecionados e analisados quanto à presença de mutações no gene *ESR1*. ESR1m foram avaliadas em amostras de tecido tumoral incluídas em blocos de parafina utilizando a técnica de reação PCR quantitativo. As mutações em *hotspots* nos códons 380, 537 e 538 do gene *ESR1* foram analisadas.

Resultados: Cento e vinte e sete pacientes foram incluídas na coorte, das quais 100 (79%) completaram a neoadjuvância, foram operadas e tiveram o escore PEPI calculado. Dentre estas pacientes, o escore PEPI variou entre 0 e 3 em 70% (70/100) e foi igual ou maior a 4 em 30% (30/100). Vinte e três pacientes foram incluídas na análise. ESR1m não foram identificadas em nenhuma das amostras dos 23 pacientes com câncer de mama em estádio inicial resistentes a hormonioterapia neoadjuvante.

Conclusão: As evidências atuais suportam a noção que existem diferentes mecanismos de resistência intrínseca ou adquirida à terapia endócrina em câncer de mama. Nosso estudo sugere que mutações no gene ESR1m não se desenvolvem em um período curto de tempo e não representam um mecanismo comum de resistência endócrina intrínseca. Portanto, ESR1m devem ser consideradas um mecanismo de resistência adquirida no contexto de câncer metastático. Pesquisas buscando identificar mecanismos de resistência endócrina intrínseca devem ser estimuladas uma vez que não existem biomarcadores com implicações na prática clínica nesta área.

Palavras chave: neoplasia de mama, câncer de mama, gene *ESR1*, mutação *ESR1*, terapia endócrina, mecanismos de resistência

ABSTRACT

Introduction: Mutations in the *ESR1* gene (ESR1m) are important mechanisms of resistance to endocrine therapy in estrogen receptor-positive (ER+) advanced breast cancer and have been recognized as a prognostic and predictive biomarker as well as a potential therapeutic target. However, published data described ESR1m as a mechanism of secondary endocrine resistance in patients with metastatic breast cancer. The role of ESR1m as a potential mechanism of primary endocrine resistance as well as whether it also occurs in tumors that are resistant to ET used early-stage disease as (neo)adjuvant has not been adequately studied. The preoperative endocrine prognostic index (PEPI) is a surrogate of endocrine sensitivity and can identify a subgroup of patients with primary resistance to ET. In this study, we evaluated the prevalence of ESR1m in tumor samples from patients with ER+ breast cancer that were resistant to neoadjuvant aromatase inhibitor therapy.

Methods: We followed a prospective cohort of postmenopausal patients with ER+ HER2- stages II-III breast cancer treated with neoadjuvant endocrine therapy (NET). Patients were treated with anastrozole for a recommended period of at least three months. Tumor samples from patients with a pattern of primary endocrine-resistant tumors (defined as a PEPI score of 4 or more) were selected and analyzed for the presence of ESR1m. ESR1m were evaluated in formalin-fixed paraffin-embedded breast cancer tissue using real-time quantitative polymerase chain reaction (RT-qPCR). Mutations in codons 380, 537, and 538 of the *ESR1* gene were analyzed.

Results: One hundred twenty-seven patients were included in the cohort, of which 100 (79%) had completed NET and underwent surgery. Among these patients, the PEPI score ranged from 0 to 3 in 70% (70/100), and 30% (30/100) had a PEPI score of 4 or more and were selected. Twenty-three patients were included in the analysis. *ESR1* mutations were not identified in any of the 23 patients with early-stage ER+ breast cancer resistant to NET,

Discussion: Growing evidence supports the notion that there are different mechanisms of primary and secondary endocrine resistance. Our study suggests that *ESR1* mutations do not evolve rapidly and do not represent a common mechanism of primary endocrine resistance in the neoadjuvant setting. Therefore, ESR1m should be considered a mechanism of acquired endocrine resistance in the context of advanced disease. Further research should be conducted to identify factors associated with intrinsic resistance to ET.

Keywords: breast neoplasm, endocrine therapy, ESR1, ESR1 mutations

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CDK	*Cyclin-dependent kinase*
CEPESG	Centro de Pesquisa da Serra Gaúcha
DNA	Ácido desoxi-ribonucleico
ESR1	Gene do receptor de estrogênio
ESR1m	Gene do receptor de estrogênio mutado
FGFR	Receptor do fator de crescimento do fibroblasto
IA	Inibidor de aromatase
IHQ	Imuno-histoquímica
IGFR	Receptor do fator de crescimento semelhante a insulina
INCA	Instituto Nacional do Câncer
LBD	*Ligand binding domain*
mTOR	*Mammalian target of rapamycin*
NGS	Sequenciamento de última geração – *next generation sequencing*
OMS	Organização Mundial da Saúde
PCR	Reação em cadeia da polimerase
PEPI	*Preoperative endocrine prognostic index*
PI3K	Fostoditil-inositol-3-quinase
PLS	Pesquisa de linfonodo sentinela
RE	Receptor de estrogênio
RP	Receptor de progesterona
SLR	Sobrevida livre de recidiva
TCGA	*The Cancer Genome Atlas*
UFRGS	Universidade Federal do Rio Grande do Sil
UNICAMP	Universidade de Campinas

INSTITUIÇÕES PARTICIPANTES

Departamento de Patologia da Universidade Federal do Rio Grande do Sul – Porto Alegre, RS

Instituto De Vita – Centro de Pesquisa da Serra Gaúcha (CEPESG) – Caxias do Sul, RS

Hospital da Mulher Prof. Dr. José Aristodemo Pinotti – Campinas, SP

Divisão de Oncologia – Laboratório de Patologia Experimental – UNICAMP – Campinas, SP

Diagnose – Laboratório de Patologia e Biologia Molecular – Caxias do Sul, RS

1 - INTRODUÇÃO

Câncer de mama

O câncer de mama é o segundo câncer mais prevalente no mundo e o mais frequente entre as mulheres, tanto em países desenvolvidos como em desenvolvimento. A Organização Mundial da Saúde (OMS), através do "GLOBOCAN Project" estimou para 2018, 1,67 milhões de casos novos e 522.000 mortes[1]. No Brasil, segundo estimativas do INCA, ocorreram 57.120 casos novos de câncer de mama em 2014, sendo a principal causa de morte por câncer entre as mulheres[2].

Quando em estágio avançado é uma doença sistêmica, progressiva e incurável e o foco do tratamento é paliativo, buscando aumentar a sobrevida e qualidade de vida do paciente. Já o câncer de mama em estádio inicial ou localmente avançado é uma doença cujo objetivo do tratamento é a cura. O tratamento deve ser multidisciplinar e normalmente é composto por cirurgia, radioterapia, quimioterapia e hormonioterapia. O tratamento deve ser individualizado conforme uma série de fatores associados ao tumor e ao paciente.

O câncer de mama é uma doença heterogênea. Esta diversidade é decorrente de múltiplas alterações genéticas e eventos moleculares. A análise genômica tem sido utilizada com sucesso para estratificação do câncer de mama em subtipos moleculares com relevantes implicações na evolução clínica bem como na avaliação dos fatores prognósticos e preditivos ao tratamento.

Subtipos moleculares de câncer de mama

Em 2000 foi publicado o primeiro trabalho com uma avaliação molecular mais ampla do câncer de mama (incluindo a análise de milhares de genes simultaneamente) que abriu novos horizontes na sua forma de classificação. Estes autores identificaram diferenças na expressão gênica (RNA mensageiro) de uma série de tumores de mama que permitia a separação dos casos em alguns subgrupos. Uma análise detalhada da lista de genes com alta e baixa expressão em cada grupo permitiu identificar os perfis de expressão gênica, ou assinaturas genéticas, característicos de cada grupo. Por fim, baseado na similaridade com algumas células do epitélio mamário os grupos foram denominados: 1) "Luminal" quando a assinatura genética se assemelhava à célula luminal do ducto mamário, 2) "Basal-símile" quando se assemelhava à célula basal, 3) "Her-2" quando os tumores apresentavam um perfil com hiperexpressão do gene Her2 ou moléculas por ele reguladas na sua via de sinalização celular. Dentro do grupo "Luminal" foi ainda possível ver subgrupos com diferenças no perfil de proliferação celular, e pelo menos dois grupos "Luminal A" e "Luminal B" persistiram nas publicações posteriores.[3]

Uma outra versão usando imuno-histoquímica (IHQ) vem sendo aplicada para traduzir o conhecimento adquirido ao nível do mRNA para o nível proteico. Através da combinação da expressão proteica dos receptores de estrógenos (RE), receptores de progesterona (RP) e Her2, três marcadores já em uso há algum tempo na propedêutica do câncer de mama, é possível identificar o grupo luminal (ER+/PR+/Her2- ou ER+/PR-/ Her2-), HER2-positivo (HER2+) e um grupo chamado triplo negativo (ER-/PR-/ Her2-). A sobreposição destes grupos com aqueles descritos originalmente não é perfeita já que a expressão gênica está sujeita a regulação pós-transcricional que faz com que o nível de expressão gênica possa ser distinto do nível de expressão proteica. Mesmo com todas as limitações esta classificação molecular através da IHQ tem ganhado popularidade porque os grupos estão associados com tratamentos específicos. O grupo luminal tem prognóstico melhor, boa resposta à hormonioterapia e pouca resposta à quimioterapia. O grupo Her2+ tem prognóstico pior, mas pela boa resposta às terapias anti-HER2 acaba por exibir sobrevida livre de evento similar aos tumores de melhor prognóstico, e o grupo triplo negativo (aproximadamente 80% de sobreposição com o grupo basal-símile) tem prognóstico pior e carece de tratamento específico[4].

Câncer de mama receptor hormonal positivo

O câncer de mama receptor de estrogênio positivo (RE+) é a principal causa de morte por câncer nas mulheres. Embora a hormonioterapia com inibidores de aromatase (IAs) suprima a função do receptor de estrogênio e reduza o risco de recorrência, a resistência terapêutica é comum na doença inicial e essencialmente inevitável no câncer de mama avançado. Mecanismos genéticos explicam por que o câncer de mama RE+ persiste, progride e causa uma doença sistêmica letal e incurável. O delineamento e os desfechos de ensaios clínicos devem ser considerados com a perspectiva que os mecanismos de resistência são heterogêneos, por isso biomarcadores e estratificação através de alterações genéticas são essenciais para melhorarmos as estratégias terapêuticas e os desfechos para os pacientes[5].

Terapia sistêmica neoadjuvante

Embora a resistência à terapia endócrina no câncer de mama avançada seja clinicamente evidente e associada com incurabilidade, a detecção (ou predição) de resistência à hormonioterapia em tumores primários antes da recidiva é uma prioridade para a pesquisa clínica, uma vez que a doença ainda está dentro de uma “janela de curabilidade”.

A terapia neoadjuvante, também chamada de neoadjuvância, consiste na administração de tratamento sistêmico (quimioterapia, hormonioterapia ou agentes biológicos) antes da cirurgia

para o câncer de mama. A terapia neoadjuvante (pré-operatória) é o tratamento padrão para o câncer de mama localmente avançado e uma alternativa aceitável à quimioterapia adjuvante em câncer de mama em estágio inicial. A resposta patológica se correlaciona com a sobrevida livre de recidiva e oferece uma oportunidade para avaliar a resposta tumoral *in vivo* e identificar biomarcadores. A possibilidade de avaliar a resposta ao tratamento com biópsias do tecido tumoral durante e no final do tratamento é o cenário ideal para a identificação de biomarcadores. Identificar biomarcadores capazes de predizer quais pacientes terão resposta patológica completa (pCR) e capazes de distinguir pacientes respondedoras das não-respondedoras representará um grande avanço na prática clínica[6].

Terapia endócrina no câncer de mama

Tratamentos direcionados contra a via do receptor de estrogênio foram as primeiras estratégias de terapia alvo em oncologia em continuam sendo o pilar do tratamento do câncer de mama RH+ ao longo de todo espectro da doença. Agentes endócrinos são utilizados em todos os estágios de câncer de mama, desde a quimioprevenção em doença não-invasora, passando pelo tratamento (neo)adjuvante em doença invasora em estágios iniciais, até o uso como agente paliativo visando benefício clínico e prolongamento de sobrevida em doença metastática. Os principais agentes endócrinos utilizados atuam diretamente na modulação (tamoxifeno) ou degradação (fulvestranto) do próprio RE ou através da diminuição dos níveis do ligante com o uso de deprivação estrogênica com supressão da função ovariana ou uso de medicamentos inibidores de aromatase[7].

Hormonioterapia neoadjuvante

A hormonioterapia neoadjuvante é uma modalidade terapêutica com crescente interesse clínico e científico. Reservada inicialmente para pacientes idosas sem condições para quimioterapia ou cirurgias radicais, a terapia endócrina pré-operatória vem sendo reconhecida como um tratamento eficaz e bem tolerado, com benefício especial em mulheres na pós-menopausa com tumores compatíveis com subtipo luminal A. Além de permitir cirurgias mais conservadoras, a hormonioterapia neoadjuvante está associada com benefícios como tolerância, perfil de segurança e custo-efetividade favoráveis. Outro potencial benefício desta modalidade é a redução dos custos de sistema de saúde, principalmente em serviços de saúde pública[8].

Ademais, a terapia endócrina neoadjuvante permite a avaliação *in vivo* da sensibilidade ou resistência ao tratamento sistêmico ainda dentro de uma janela de curabilidade, permitindo o direcionamento de pacientes com tumores resistentes para tratamento alternativos através desta importante ferramenta científica. Estudos de biomarcadores preditivos são de fundamental importância na busca por uma oncologia mamária personalizada[9].

Ao contrário dos tumores triplo negativos, os tumores luminais são sensíveis à hormonioterapia e apresentam baixa resposta à quimioterapia neoadjuvante, com taxas de pCR que variam de 6-8%. Para esses tumores, a hormonioterapia neoadjuvante representa uma alternativa com baixa toxicidade. Estudos que compararam a quimioterapia neoadjuvante à hormonioterapia neoadjuvante demonstraram taxas semelhantes de reposta clínica, variando de 48-89% e 64-85%, respectivamente. Em um estudo randomizado que comparou 4 ciclos de quimioterapia com três meses de hormonioterapia, não houve diferença nas taxas de resposta tumoral entre os dois grupos (63.6% vs. 64.5%; $p > 0.5$); entretanto, as taxas de cirurgia conservadora foram superiores no grupo da hormonioterapia (33% vs. 24%; $p = 0.058$)[10]. Apenas um estudo randomizado investigou o benefício da hormonioterapia pré-operatória comparado com cirurgia apenas, e demonstrou uma taxa de resposta no grupo da terapia neoadjuvante de 83% e aumento das taxas de cirurgia conservadora (OR 2.31; 95%CI 1.22-4.37)[8].

A aplicação da hormonioterapia neoadjuvante como ferramenta científica na busca de biomarcadores preditivos é crescente. Um dos exemplos mais estudados é o Ki67, marcador avaliado por IHQ que está associado com replicação celular. Pacientes com queda do Ki67 após exposição à hormonioterapia tiveram uma diminuição estatisticamente significativa da chance de recidiva. Em uma análise multivariada do estudo PO24, quatro fatores tiveram valor prognóstico em relação à recidiva e morte: o tamanho tumoral, o status linfonodal e expressão de Ki67 e RE na peça cirúrgica. Um escore prognóstico chamado PEPI (Preoperative Endocrine Prognostic Index) foi desenvolvido baseado nesses dados e permitiu identificar um subgrupo de pacientes com excelente prognóstico, aqueles com escore 0 ou 1. Estes pacientes provavelmente não se beneficiariam de quimioterapia adjuvante uma vez que a terapia endócrina isolada parece controlar adequadamente a doença[11].

A medicina personalizada demanda procura por biomarcadores preditivos e prognósticos que possam ser aplicados para pacientes individuais buscando informações para um manejo clínico otimizado. O objetivo deste estudo é demonstrar que a utilização da neoadjuvância pode identificar biomarcadores associados com padrão de sensibilidade ou resistência ao tratamento sistêmico e potencialmente novos alvos para o desenvolvimento racional de novas terapias[12].

A via do receptor do estrogênio (RE)

O receptor de estrogênio (RE), uma proteína codificada pelo gene *ESR1*, é expressa em aproximadamente 70% dos cânceres de mama. A expressão de RE é uma das características definidoras na classificação do subtipo tumoral e na definição terapêutica. Um grande número de experimentos e de pesquisas clínicas estabeleceu o papel fundamental do RE e do seu ligante, o estrogênio, no desenvolvimento do tecido mamário normal e na etiologia e na progressão do câncer de mama.

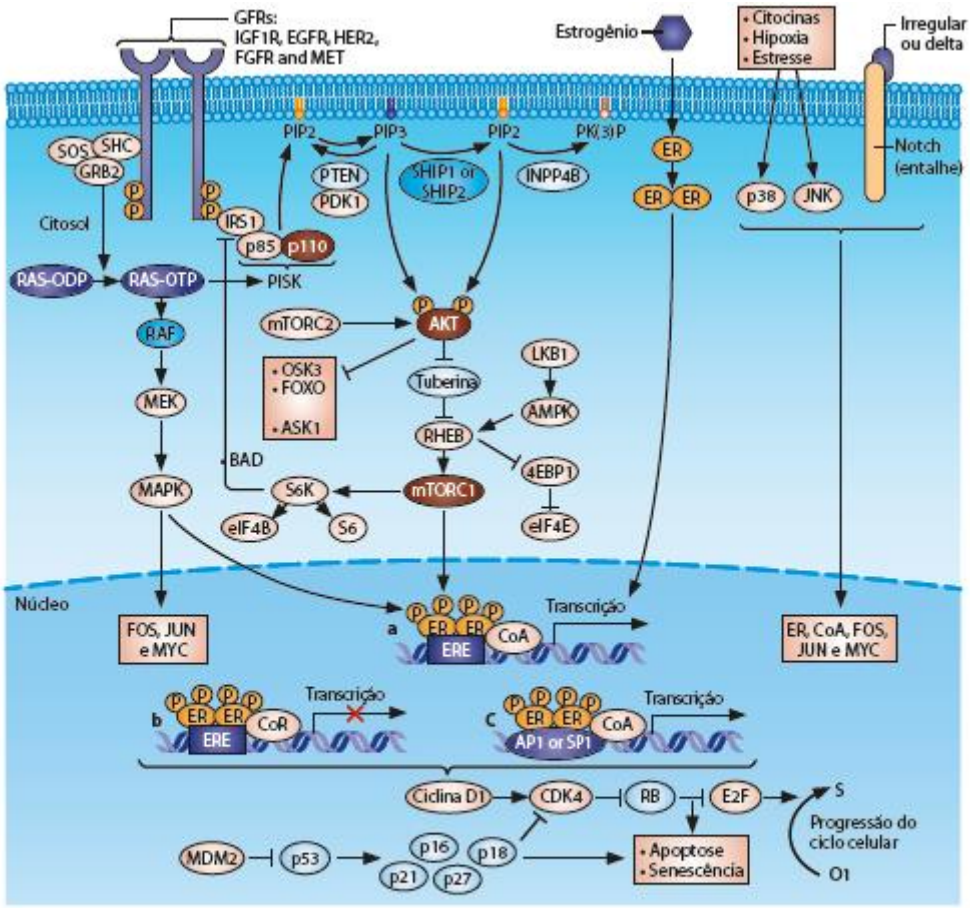
O RE é uma proteína predominantemente nuclear que funciona como um fator de transcrição dependente de um ligante. A ligação do RE ao estrógeno circulante desencadeia um número de eventos que resultam em ativação transcricional do RE e leva a mudanças conformacionais na região do Ligand Binding Domain (LBD) que permitem a ligação do RE as sequências específicas de DNA (elementos de resposta ao estrogênio -ERE) e a interação com proteínas co-ativadoras e co-repressoras que regulam a transcrição de genes estrógeno-responsivos e que são importantes em vários processos fisiológicos e patológicos, incluindo a carcinogênese e progressão tumoral[13].

Mecanismos de resistência à terapia endócrina em câncer de mama

O cancer de mama RE+ é o mais comum e a principal causa de mortalidade por esta doença. A terapia endócrina é o pilar mais importante do tratamento do cancer de mama RE+ em todos os estágios da doença. Apesar dos benefícios significativos com a terapia endócrina, os tumores de mama são conhecidos por desenvolverem evolução genômica durante o tratamento com a aquisição de novas mutações que conferem resistência à terapia. Portanto, uma significativa proporção de pacientes com câncer em estágio inicial submetido a tratamento com intuito curativo sofrerá recidiva da doença apesar de tratamento locorregional e terapia sistêmica adjuvante. No contexto de doença metastática, ainda que a maior parte dos pacientes tenha benefício e controle de doença inicialmente com a terapia endócrina, o desenvolvimento de recorrência e a progressão da doença invariavelmente acontece, fazendo com que o câncer de mama metastático seja uma doença sistêmica, progressiva e incurável[14].

Estes mecanismos de doença são múltiplos e possuem complexas interações entre si. Podem envolver a interação da via do RE com proteínas na membrana das células tumorais (como receptores de fatores de crescimento como HER2 e IGFR), a interação da via do RE com

a ativação de cascatas de proteínas intracitoplasmáticas como PI3K/Akt/mTOR e também a modulação de proteínas intranucleares envolvidas na regulação do maquinário do ciclo celular. Além disso, existem uma série de alterações moleculares no próprio gene *ESR1* que codifica o receptor de estrogênio e que podem levar a ativação constitucional desta via e consequente proliferação celular e progressão tumoral[15].



Adaptado de: Ma C, et al., 2015.[3]

Figura 1 – Interação entre as diversas vias associadas com mecanismos de resistência à terapia endócrina do câncer de mama[3]

Mutações do gene do receptor do estrogênio

A mutação no gene do receptor de estrogênio ESR1 foi descrita pela primeira vez em 1997. Entretanto, estudos subsequentes realizados em câncer de mama primário não identificaram mutações frequentes em ESR1, e o potencial impacto clínica de ESR1m foi subestimado. Estudos genômicos em larga escala, como o The Cancer Genome Atlas (TCGA) trouxeram novas

perspectivas e ampliaram os conhecimentos sobre a heterogeneidade e a complexidade genômica do câncer de mama. Apesar do papel importante do RE em tumores luminais, dados do TCGA indicaram que ESR1m estavam presentes em apenas 0,5% dos tumores dentre 962 amostras de câncer de mama.. Somente em 2013 que uma série de estudos utilizando sequenciamento de última geração do DNA renovaram o interesse demonstrando uma alta prevalência (11 a 55%) de mutaçãoes no LBD do gene *ESR1* em pacientes expostos a tratamento com IAs, mas não em pacientes virgens de tratamento endócrino[16].

Mutações no *ESR1* são raramente encontradas em pacientes não tratadas com terapia endócrina, o que sugere seleção de clones resistentes mais raros ou a aquisição de mecanismos de resistência secundários decorrentes da pressão do tratamento endócrino. As mutações de *ESR1* são recorrentes, sendo mais frequentemente detectadas nos códons 537 e 538 do LBD, levando a ativação constitutiva do receptor independente de ligante[17]. A prevalência de ESR1m variou entre 11 e 54% nas series publicadas, muito em decorrência da diferença entre as populações estudadas[181920]. Dados mais recentes avaliando biópsias liquídas em pacientes com carcinoma de mama avançado RH+ refratário a IAs sugere uma prevalência entre 20 e 30%[16].

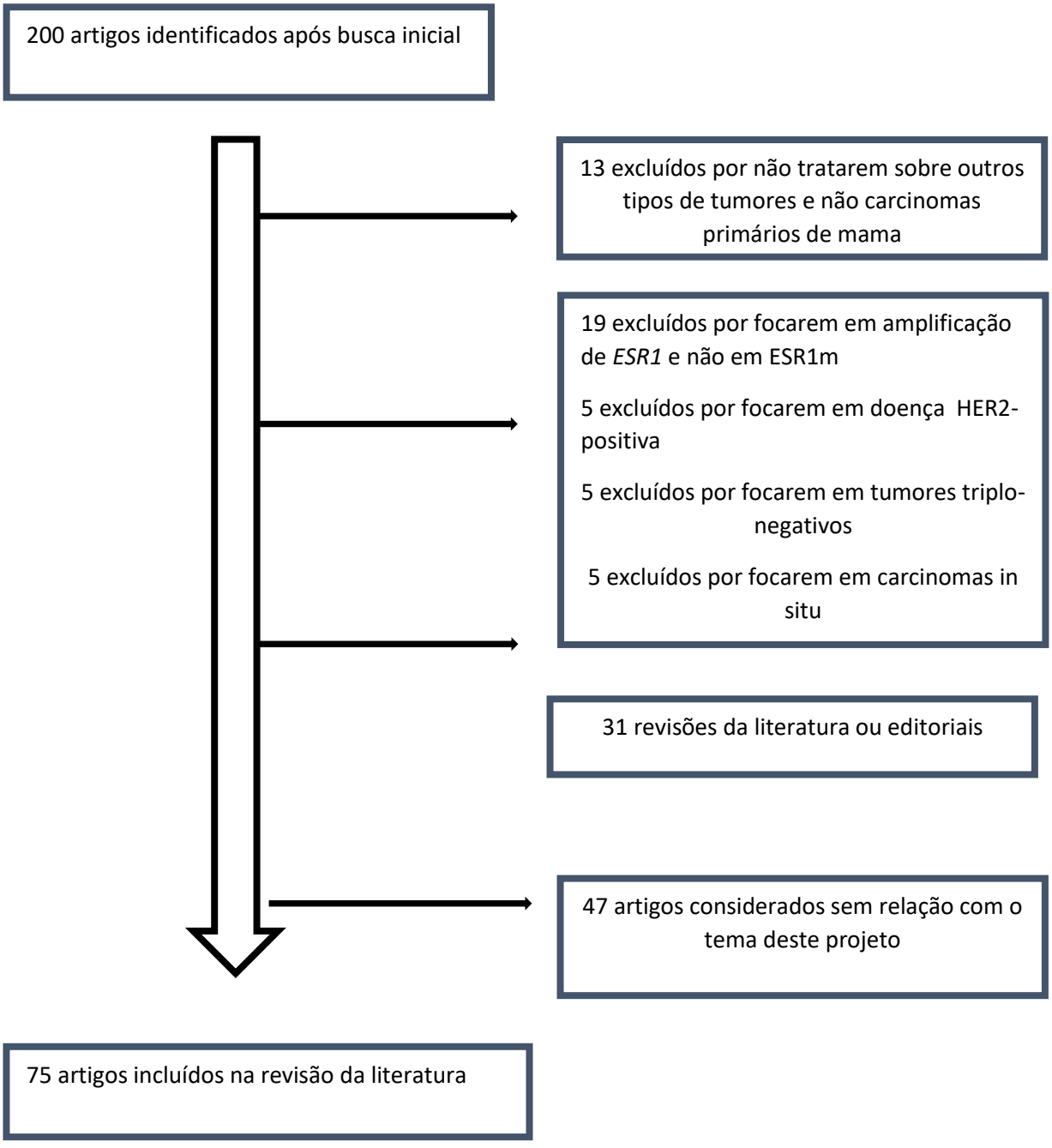
A terapia alvo dirigida aos clones com mutação de *ESR1* é um conceito atrativo e dados pré-clínicos e clínicos sobre o desenvolvimento de tratamentos e estratégias terapêuticas que visam inibir os clones mutantes tem o potencial de melhorar desfechos clínicos. Atualmente, a presença de mutação de *ESR1* pode ser considerada um biomarcador prognóstico negativo associado com piora de desfechos de sobrevida livre de progressão e sobrevida global. O potencial de ESR1m como biomarcador preditivo vem sendo desenvolvido rapidamente. Baseado em dados atuais, a presença de ESR1m está associada com piores desfechos com tratamento com IAs. Benefícios associados com uso de outros agentes endócrinos como fulvestranto parecem ser semelhantes. O uso de combinação com agentes como inibidores de mTOR e inibidores de CDK4/6 parece não ser influenciado pela presença ou ausência de ESR1m[1321].

Esforços futuros nesta área de pesquisa devem promover que ESR1m seja utilizado como um biomarcador integral em ensaios clínicos de câncer de mama HR+ e que seja testado prospectivamente como fator de estratificação, como estratégias de estudos adaptativos e como alvo terapêutico no desenvolvimento de novas estratégias para superar mecanismos de resistência à terapia endócrina no câncer de mama.

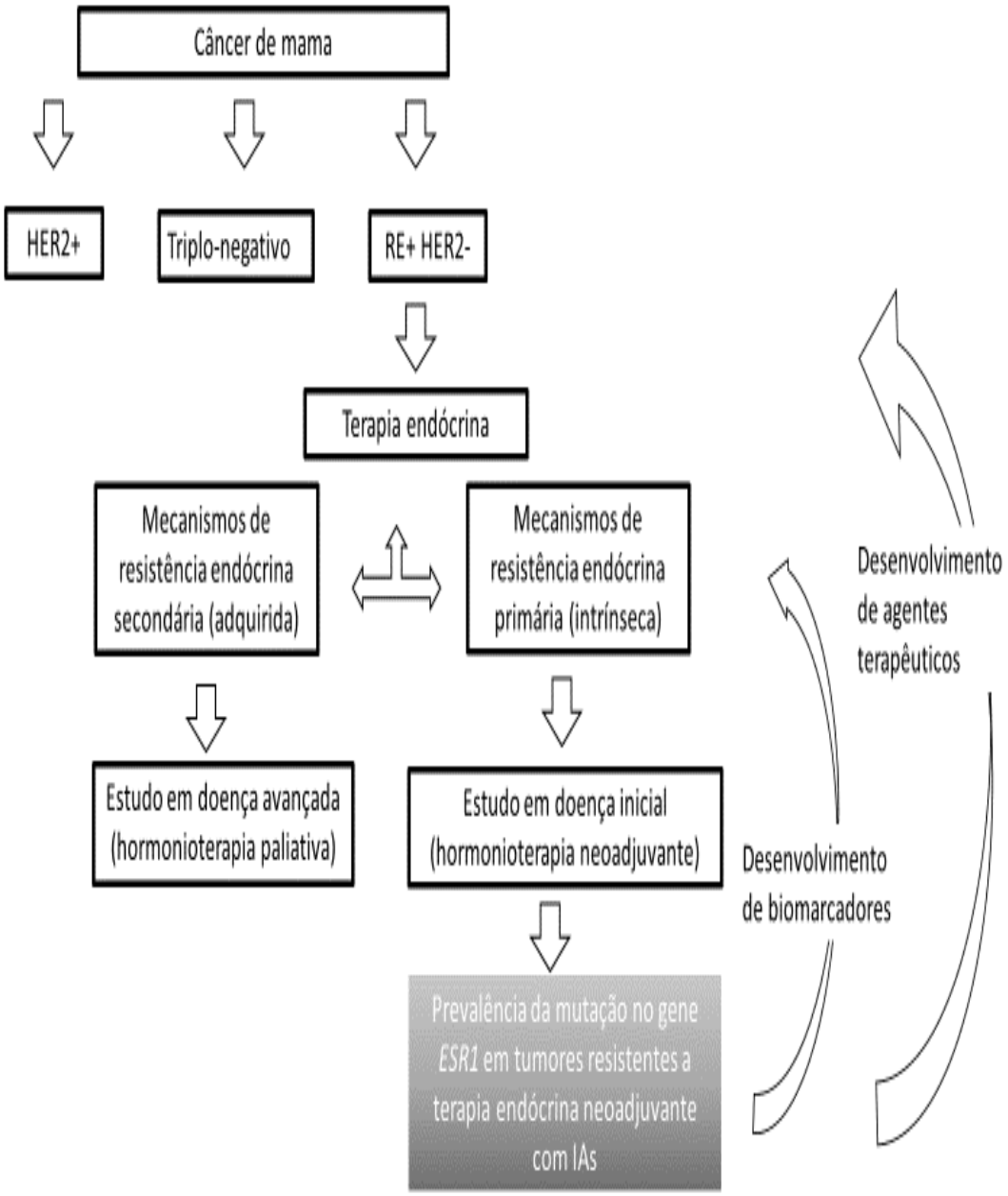
2 - REVISÃO DA LITERATURA

Metodologia da revisão de literatura

A revisão da literatura foi atualizada e focada especificamente nos aspectos relacionados ao papel da mutação do gene *ESR1* como mecanismos de resistência à terapia endócrina em câncer de mama. A estratégia de busca envolveu a base de dados Pubmed. Foram realizadas buscas através dos termos “breast cancer” E “ESR1 mutation’ e suas combinações. ("breast neoplasms"[MeSH Terms] OR ("breast"[All Fields] AND "neoplasms"[All Fields]) OR "breast neoplasms"[All Fields] OR ("breast"[All Fields] AND "cancer"[All Fields]) OR "breast cancer"[All Fields]) AND (esr1[All Fields] AND ("mutation"[MeSH Terms] OR "mutation"[All Fields])). A busca resultou em 200 artigos científicos publicados, dentre os quais 188 foram selecionados após leitura criteriosa dos títulos abstracts (conforme descrito no fluxograma abaixo – Figura 2)



3 – MARCO CONCEITUAL



4 - JUSTIFICATIVA

Câncer de mama é uma doença heterogênea que é composta por diferentes subtipos clínicos, histológicos e molecular. Os tumores com receptor hormonal positivo (RH+) representam a forma mais comum de câncer e esse subtipo é o maior responsável por mortes por esta doença. A terapia endócrina é prescrita para virtualmente todos os pacientes com câncer de mama RH+. Entretanto, devido a uma complexa variedade de mecanismos de resistência, uma significativa proporção dos pacientes com doença em estágio inicial apresenta recidiva apesar do tratamento locorregional e terapia sistêmica adjuvante. No contexto metastático, ainda que muitas pacientes tenham benefício clínico e controle de doença por períodos prolongados, resistência e progressão de doença invariavelmente ocorrem. Tumores de mama apresentam evolução genômica durante o curso da doença com a aquisição de novas mutações que conferem resistência às terapias. A descoberta recente de mutações recorrentes no gene que codifica o receptor de estrogênio – *ESR1* – e que estão associadas a ativação constitucional independente de ligante do RE, trouxe novas perspectivas no entendimento de mecanismos de resistência à terapia endócrina em câncer de mama

A terapia neoadjuvante do câncer de mama é uma modalidade que permite ao mesmo tempo proporcionar ao paciente o melhor tratamento multidisciplinar disponível como serve como plataforma para estudo de biomarcadores prognósticos e preditivos associados a mecanismos de resistência ao tratamento sistêmico. Isto permitirá uma maior personalização do tratamento oncológico do câncer de mama além de servir como base para o desenvolvimento de pesquisa clínica com terapias alvo.

A utilização da resposta patológica completa (pCR) como único indicador de resposta ao tratamento neoadjuvante subestima o benefício clínico do tratamento em termos de sobrevida livre de progressão. O uso de medidas de doença residual pode aumentar o poder de ensaios clínicos neoadjuvantes e melhorar as estimativas de chance de recorrência. Existe um grande interesse em desenvolver mais informações prognósticas da doença residual através da avaliação de sua extensão e comportamento biológicos. Diferentes sistemas de classificação podem resultar em diferentes estimativas do risco de recorrência. Sistemas que combinam informações clínicas, patológicas e moleculares antes e após a neoadjuvância trazem a possibilidade de incorporar informações sobre a doença inicial virgem de tratamento e a doença residual após tratamento, permitindo avanço no conhecimento da heterogeneidade e dos mecanismos de resistência no câncer de mama RH+.

Este estudo é o braço dedicado à análise do subgrupo de tumores com receptor hormonal positivo dentro de um novo projeto multicêntrico de estudos em tratamento neoadjuvante de câncer de mama. Uma das metas é a padronização entre as instituições participantes em termos de modalidades terapêuticas e de avaliação histopatológica e genética, além da criação de banco de dados e parceria institucional que servirão como fonte para pesquisas posteriores.

5 - OBJETIVOS

5.1 - Objetivo primário

- Avaliar a prevalência da mutação do gene *ESR1* em pacientes com câncer de mama submetidas a terapia endócrina neoadjuvante com inibidores de aromatase e que apresentem doença residual com PEPI Score superior a 3.

5.2 - Objetivos secundários

- Comparar a prevalência de ESR1m em pacientes com tumores resistentes à hormonioterapia neoadjuvante com a taxa de prevalência de ESR1m em tumores metastáticos resistentes a hormonioterapia na nossa população.
- Descrever as taxas de escore PEPI em uma coorte prospectiva de pacientes com câncer de mama tratadas com hormonioterapia neoadjuvante
- Descrever as características clínicas e epidemiológicas além dos resultados em termos de patologia e IHQ das pacientes com câncer de mama com perfil de resistência a terapia endócrina neoadjuvante

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7 – ARTIGO PRINCIPAL

TITLE: ESR1 mutations are not a common mechanism of endocrine resistance in patients with estrogen-receptor positive breast cancer treated with neoadjuvant aromatase inhibitor therapy

Tomás Reinert[1,2,3], Susana Ramalho[4], Vivian Castro Antunes de Vasconcelos[4], Leonardo Roberto Silva[4], Ana Elisa Ribeiro da Silva[4], Camila Annicchino de Andrade[4], Maria Beatriz de Paula Leite Kraft[4], Guilherme Portela Coelho[5], Jovana Mandelli[5], Monique Binotto[2], Cesar Cabello[4], Geisilene Russano de Paiva Silva[6], José Bines[7], Carlos H Barrios[3], Matthew J Ellis[8], Marcia Silveira Graudenz[1]

AFFILIATIONS:

1 – Postgraduate Program in Medical Sciences, Universidade Federal do Rio Grande do Sul (UFRGS) – Porto Alegre, Brazil

2 – Centro de Pesquisa da Serra Gaucha (CEPESG) – Caxias do Sul, Brazil

3 – Latin American Cooperative Oncology Group (LACOG) – Porto Alegre, Brazil

4 – Faculty of Medical Sciences, Department of Obstetrics and Gynecology, State University of Campinas (UNICAMP) – Campinas, Brazil

5 – Diagnose Patologia e Biologia Molecular – Caxias do Sul, Brazil

6 – Laboratory of Molecular and Investigative Pathology – LAPE, Faculty of Medical Sciences, State University of Campinas (UNICAMP) – Campinas, Brazil

7 – Instituto Nacional do Câncer (INCA – HCIII) – Rio de Janeiro, Brazil

8 – Lester and Sue Smith Breast Cancer Center – Baylor College of Medicine – Houston, US

CORRESPONDING AUTHOR

Tomás Reinert

CEPESG

Rua Vinte de Setembro 2304, 701 – Caxias do Sul, RS, Brazil

tomasreinert@hotmail.com

ARTICLE TYPE: Research Article

TARGET JOURNAL: Breast Cancer Research and Treatment

KEYWORDS: breast cancer; endocrine therapy; *ESR1*; ESR1 mutations; neoadjuvant, aromatase inhibitors;

ABSTRACT

Introduction: Mutations in the *ESR1* gene (ESR1m) are important mechanisms of resistance to endocrine therapy in estrogen receptor-positive (ER+) advanced breast cancer and have been recognized as a prognostic and possibly a predictive biomarker as well as a potential therapeutic target. Published data describe ESR1m as a mechanism of secondary endocrine resistance in patients with metastatic breast cancer. However, the role of ESR1m as a potential mechanism of primary endocrine resistance as well as whether it also occurs in tumors that are resistant to ET administered in early-stage disease as (neo)adjuvant has not been adequately studied. The preoperative endocrine prognostic index (PEPI) combining Ki67 score, ER Allred score, tumor size, and nodal status after neoadjuvant endocrine therapy is a surrogate of endocrine sensitivity and can identify a subgroup of patients with primary resistance to ET. In this study, we evaluated the prevalence of ESR1m in tumor samples from patients with ER+ breast cancer resistant to neoadjuvant aromatase inhibitor therapy.

Methods: We followed a prospective cohort of postmenopausal patients with ER+ HER2- stages II-III breast cancer treated with neoadjuvant endocrine therapy (NET). Patients were treated with anastrozole for a recommended period of at least three months. Tumor samples from patients with a pattern of primary endocrine-resistance (defined as a PEPI score of 4 or more) were identified and analyzed for the presence of ESR1m. ESR1m were evaluated in formalin-fixed paraffin-embedded breast cancer tissue using real-time quantitative polymerase chain reaction (RT-qPCR). Mutations in codons 380, 537, and 538 of the *ESR1* gene were analyzed.

Results: One hundred twenty-seven patients were included in the cohort, of which 100 (79%) had completed NET and underwent surgery. Among these patients, the PEPI score ranged from 0 to 3 in 70% (70/100), while 30% (30/100) had a PEPI score of 4 or more. Twenty-three of these patients were included in the analysis. *ESR1* mutations were not identified in any of the 23 patients with early-stage ER+ breast cancer resistant to NET.

Discussion: Growing evidence supports the notion that there are different mechanisms for primary and secondary endocrine resistance. Our study suggests that *ESR1* mutations do not evolve rapidly and do not represent a common mechanism of primary endocrine resistance in

the neoadjuvant setting. Therefore, ESR1m should be considered a mechanism of acquired endocrine resistance in the context of advanced disease. Further research should be conducted to identify factors associated with intrinsic resistance to ET.

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INTRODUCTION

Estrogen receptor-positive (ER+) breast cancer is the most prevalent breast cancer subtype. Endocrine therapy (ET), a targeted treatment to the estrogen receptor (ER) pathway, remains the mainstay of treatment in all stages of the disease[22]. Nevertheless, endocrine resistance associated with disease progression remains an important challenge[2314]. Mutations of the *ESR1* gene, which encodes the ER protein, are recognized as a mechanism of endocrine resistance to an increasing extent[17].

Breast tumors undergo genomic evolution, and *ESR1* mutations (ESR1m) have been described in 9-40% of patients with advanced ER+ breast cancer resistant to aromatase inhibitors[1424172526]. In Brazilian patients with visceral metastasis of ER-positive HER2-negative breast cancer, our group found the presence of ESR1m in 25% of the cases[27]. In the metastatic setting, the presence of ESR1m is a biomarker of worse prognosis. The function of ESR1m as a predictive biomarker as well as a potential therapeutic target is being studied[28]. However, the role of ESR1m as a possible mechanism of ET resistance in the early-stage disease is unclear. Regardless of the extensive research that is being conducted in this field, several questions remain unanswered about ESR1m, such as whether it is associated with resistance to aromatase inhibitors (AIs) used in the (neo)adjuvant setting.

Neoadjuvant endocrine therapy (NET) is a therapeutic approach that is being increasingly explored, not only to allow less extensive surgery but also as a scientific tool, exploring biomarkers to predict outcomes[9]. The preoperative endocrine prognostic index (PEPI) is a surrogate of endocrine sensitivity and identifies a subgroup of patients with primary resistance to ET[911].

We aimed here to evaluate the potential role of ESR1m as a mechanism of resistance in postmenopausal patients with stage II and III breast cancer treated with NET with a high PEPI score as a surrogate for primarily endocrine-resistant biology.

METHODS

We conducted a prospective cohort of postmenopausal patients with ER+ HER2- stages II-III breast cancer treated with NET in two institutions. The population included postmenopausal women presenting with stage II and III ER-positive Her2-negative breast cancer. Protocols of diagnosis, treatment, and follow-up of patients were standardized and based on major international guidelines. These procedures are not part of the study.

Eligible patients were treated with NET with anastrozole for a recommended period of at least three months, followed by surgery. Pathological and immunohistochemistry protocols were standardized and followed ASCO/CAP guidelines and the international consensus of pathologic assessment of the breast and axilla after preoperative therapy[10]. For all patients that underwent surgery, the PEPI Score was calculated (described in Table 1). Tumor samples from patients with a pattern of primary endocrine-resistant tumors (defined as a PEPI score of 4 or more) were selected and analyzed for the presence of ESR1m.

TABLE 1

Surgical factors	**RFS HR**	**PEPI Score**
Tumor Size		
T1/T2	-	0
T3/T4	2.8	3
Node status		
Negative	-	0
Positive	3.2	3
Ki67 level		
0-2.7%	**-**	**0**
>2.7%-7.3%	1.3	1
>7.3%-19.7%	1.7	1
>19.7%-53.1%	2.2	2
>53.1%	2.9	3

ER status		
Negative	2.8	3
Positive	0	0

Legend: The preoperative endocrine prognostic index (PEPI) score following 4–6 months of neoadjuvant AI or other endocrine therapy provides another strategy to identify endocrine-sensitive versus endocrine-resistant tumours in the early-stage setting. A PEPI score of 0 (pT1/2, node-negative (N0), Ki67<2.7%, oestrogen receptor-positive (ER^+)) is being investigated prospectively as a surrogate of endocrine therapy-sensitive disease that does not need chemotherapy, and a PEPI of >0 identifies patients with an increased risk of relapse. The hazard ratio (HR) of each surgical factor for relapse-free survival (RFS) and assigned PEPI points based on the data from the P024 trial are shown in the figure. Adapted from Ma CX et al[14])

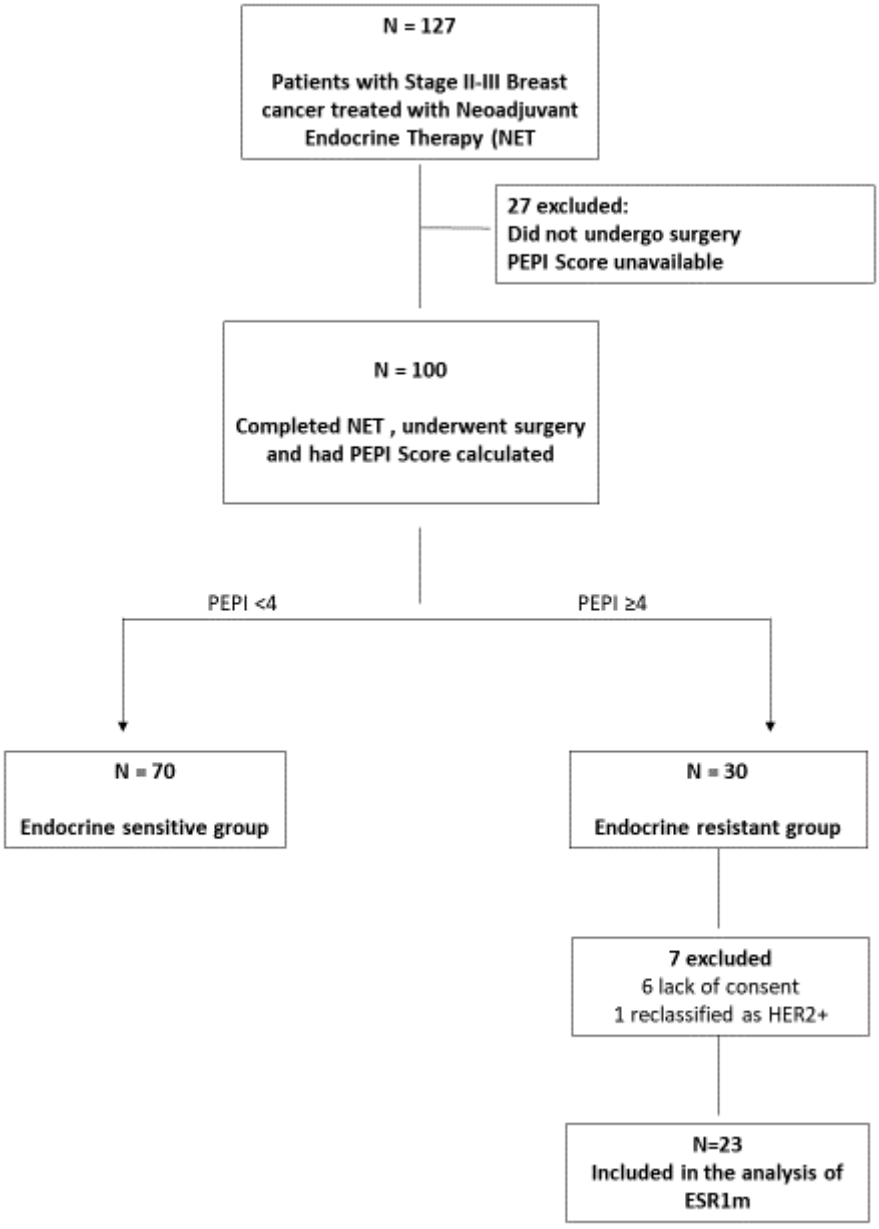
ESR1m were evaluated in formalin-fixed paraffin-embedded (FFPE) breast cancer tissue using real-time quantitative polymerase chain reaction (RT-qPCR). In each sample, the tumor area was marked by the pathologist, followed by the extraction of the genetic material (DNA) with the Wizard© Genomic DNA Purification kit (Promega). DNA was quantified using Qubit Fluorometric Quantitation (Thermo Fischer Scientific), and 20ng/µl was the threshold for the analysis of the mutation. The reactions were performed with the equipment 7500 Fast Real-Time PCR System using TaqMan Genotyping Master Mix, primers, and TaqMan© probes and following all recommendations of the manufacturer. The analyzed mutations were Y537N, Y537C, Y537S, E380Q, D538G. To detect the presence of the mutation, a Taqman© reference probe was used, followed by analysis in the 7500 Software v2.06 (Thermo Fischer Scientific).

Statistical analysis was performed using Statistical Package for Social Sciences 22.0 (SPSS). Categorical variables are described by frequencies and percentages. The primary endpoint is the prevalence of ESR1m. For the primary endpoint, the point estimate is presented together with the exact 95% CI. Continuous variables are described by means and standard deviation or median, depending on Shapiro-Wil test analysis. Correlations between categorical variables were analyzed by the chi-square test. Differences between means are analyzed by the student T-test. This project was reviewed and approved by the institutional review board (Ethical Committee) of both institutions.

RESULTS

One hundred twenty-seven patients were included in the cohort, of which 100 (79%) completed NET and surgery. Among these patients, the PEPI Score ranged from 0 to 3 in 70% (70/100), and 30% (30/100) had a PEPI score of 4 or more and were selected. Twenty-three patients were included in the analysis (6 did not consent or were lost to follow-up, and one was found to be HER2-positive in the surgical sample). These data are summarized in Figure 1.

FIGURE 1



Legend: Flowchart for the inclusion in the analysis of ESR1m in endocrine resistant tumors

Table 2 summarizes the most important characteristics of the patients and biopsy findings at initial diagnosis. Tumors classified as primarily resistant to ET (PEPI Score of four or more) were selected and had their tumors analyzed for ESR1m. In our analysis we median age was 70 years old. There was a predominant of ductal carcinoma (86,9%), T1-T2 tumors (60,8%) and the majority of our patients had clinical positive axillary nodes (56.5%) before treatment and had a Ki67 index between 7.4% and 19.7%. Table 3 describes the results in terms of PEPI Score and pathological and IHC evaluation. On pathological analysis (after NET) most of tumors were T1/T2 (73.9%), had a compromised axilla (91.3%) maintained ER positivity (95.6%) and had intermediate levels of Ki67.

Table 2 – Patients characteristics, pathology and IHC results (initial biopsy sample)	**(n=23)**
Age	Median 70 (54-84)
Histology	Ductal: 20 (86.9%) Lobular: 3 (13.1%)
Clinical tumor size	cT1/T2: 14 (60.8%) cT3/T4: 9 (39.2%)
Clinical lymph node status	Negative (cN+): 10 (43.4%) Positive (cN+): 13 (56.5%)
Ki67 level – baseline	<2.7%: 2 (8.6%) 2.8%-7.3%: 1 (4.3%) 7.4%-19.7%: 12 (52.1%) 19.8-53.1%: 8 (34.7%) >53.2%: 0
Duration of NET	Median 22 (4-35)

Table 3 – PEPI Score, pathology and IHC results after NET	(surgical sample)
PEPI Score	IV: 16 (69.5%)
	V: 2 (8.6%)
	VI: 3 (13%)
	VII: 2 (8.6%)
Pathological tumor size – surgical specimen	ypT1/T2: 17 (73.9%)
	ypT3/T4: 6 (26%)
Pathological lymph node status	Negative (ypN-): 2 (8.6%)
	Positive (ypN+): 21 (91.3%)
ER status, Allred score	0-2: 1 (4.3%)
	3-8: 22 (95.6%)
Ki67 level – surgical specimen	<2.7%: 5 (21.7%)
	2.8%-7.3%: 5 (21.7%)
	7.4%-19.7%: 11 (47.8%)
	19.8-53.1%: 1 (4.3%)
	>53.2%: 1 (4.3%)

The median duration of NET was 22 weeks (range 4-35 weeks). All samples of tumor tissue from the surgical specimens after NET were evaluable. Quantification of DNA extraction and reference gene cycle threshold values confirmed that the material was adequate for the analysis. *ESR1* mutations were not identified in any of the 23 samples. Therefore, the prevalence of ESR1m in patients with early-stage ER+ HER2-negative breast cancer that were intrinsically resistant to NET is 0% (95% IC – 0%-12.2%).

DISCUSSION

Our study reports that differently than in AI-resistant metastatic breast cancer, ESR1m are not a common mechanism of resistance in tumors with primary endocrine resistance in the neoadjuvant setting. This finding has implications for the future development of ESR1m as a biomarker and therapeutic target.

Estrogen receptor-positive (ER+) tumors are the most common form of breast cancer and responsible for most of the deaths caused by this disease[29]. ET is the mainstay of ER+ breast cancer therapy in all stages of the disease. In the metastatic disease setting, the use of ET agents

is associated with clinical benefit in the majority of patients. Nonetheless, disease progression associated with a complexity of mechanisms of resistance remains a significant challenge[29]. In early-stage disease, a considerable proportion of patients will develop disease recurrence despite the use of curative-intent treatment with a combination of therapies such as surgery, chemotherapy, radiation, and adjuvant hormone therapy.

ER, a protein encoded by the *ESR1* gene, is expressed in the majority of breast cancers. ER expression is one of the defining features for classifying tumor subtype and assigning therapeutic strategies in breast cancer. Translational and clinical research have established the fundamental role of ER and its hormonal ligands in normal mammary gland development and in the etiology and progression of breast cancer. Estrogen binding triggers several events resulting in conformational changes in the LBD, activation of the receptor and allowing the ligand-receptor complex to bind to specific DNA sequences while interacting with co-repressor and co-activator proteins to regulate the transcription of estrogen-responsive genes. Breast tumors undergo genomic evolution during therapy, with the development of new alterations that confer resistance to treatment. *ESR1* is known to undergo LBD mutations, gene amplification or translocations that are potential mechanisms of resistance to ET.[30] [18] [31] ESR1m are most commonly missense mutations clustered in codons 537 and 538 of the LBD. The most prevalent *ESR1* point mutations are Y537S and D538G, while several others have been identified at significantly lower frequencies. ESR1m have been consistently associated with inferior outcomes and are being evaluated as predictive biomarkers to help guide therapeutic decisions[32]. At the same time, the development of specific targeted therapies directed to *ESR1*-mutant clones is an appealing concept with interesting preclinical data already published and promising clinical work in progress[33]. [34]

The role of ESR1m has been mostly studied in patients with advanced breast cancer with disease progression after AI used in the metastatic setting, usually presenting with a clinical pattern of acquired (secondary) ET resistance. Nevertheless, the potential role of ESR1m in patients with early-stage breast cancer treated with (neo)adjuvant ET has not been adequately studied. While ESR1m have been identified in patients with metastatic disease, their presence in primary tumors is very low[35]. Understanding mechanisms of intrinsic (acquired) endocrine resistance is of great importance, given that it could lead to the development of biomarkers and therapeutic agents that could be incorporated in curative-intent treatment of breast cancer.

NET is being increasingly used not only as a clinical tool but also as a scientific tool for the study of tumoral patterns of intrinsic sensitivity and resistance to endocrine therapy. NET has been indicated as a clinical tool for tumor downstaging to allow breast-conserving surgery.

Additionally, NET allows an *in vivo* observation of the response to estrogen deprivation therapies. Ki67 expression at surgery following neoadjuvant AI therapy, in addition to three other independent prognostic factors, pathological tumor size, lymph node status and ER level, has been incorporated into a prognostic tool: the preoperative endocrine prognostic index (PEPI) described in Figure 1, which further distinguishes endocrine therapy-sensitive from therapy-resistant tumors to guide systemic adjuvant therapy. In the P024 and IMPACT neoadjuvant endocrine therapy trials, the early relapse rate was very low in patients with a PEPI score of 0[36]. The rate of PEPI-0 tumors in neoadjuvant AI therapy trials ranges from 17% to 37%[36]. These patients represent a low-risk population that could potentially receive adjuvant endocrine monotherapy and be spared from chemotherapy.[14] PEPI scores above zero (either because of high disease burden at surgery, or high Ki67 levels, or both) were associated with an incremental increase in the risk of relapse.

We compared these findings with a study from our group using the same methodology in patients with advanced disease where *ESR1* mutations were detected in 25% (n=32) of patients with visceral metastasis of ER+ breast cancer resistant to endocrine therapy[27] and found a statistically significant association of the presence of ESR1m and metastatic disease in comparison with tumors resistant to AIs used in the neoadjuvant setting (p 0.01, Fisher´s exact test).

Therefore, the discovery of biomarkers associated with resistance to NET as well as adjuvant ET remains an unmet need. Our data reinforce the notion that mutations in the *ESR1* gene do not seem to evolve rapidly and are probably mechanisms of secondary resistance to ET. Ongoing studies are evaluating a variety of potential mechanisms of primary endocrine resistance, such as defects in the DNA repair machinery[37] and aberrant FGFR signaling[38].

Our study has limitations, and we recognize that the detected prevalence of ESR1m can be underestimated given the fact that a PCR-based methodology was used and only specific mutations in the most commonly mutated codons were analyzed, therefore cases with mutations in different codons of the *ESR1* gene potentially detectable with next-generation sequencing technologies were not identified[3940]. Additionally, and despite the challenge of conducting a prospective cohort of neoadjuvant endocrine therapy in breast cancer with more than one hundred patients, we acknowledge that the relatively low sample size of 23 cases of tumors with a PEPI Score of 4 or more is a limitation and highlights the challenges of studying primary endocrine resistance. Furthermore, our patients were treated with median 3 months of NET. Therefore, we cannot exclude that resistance documented after longer treatment durations (6-12 months) could not be related to receptor mutations, but in these cases, it would

be expected that patients would have at least stable disease or a degree of response to allow for continuation of treatment. This would make it unlikely to relate ESR1m with primary resistance.

Among the strengths of our study, we highlight the conduction of a well-designed prospective cohort including 127 breast cancer patients that were treated with standard of care NET and whose tumors were evaluated with uniform procedures of pathology and immunohistochemistry. Additionally, we used a validated methodology for *ESR1* determination and we were able to perform a prespecified comparison in two different settings (neoadjuvant and metastatic). Despite the relatively low sample size we demonstrated that the prevalence of ESR1m in NET-resistant tumors is very low (95% of chance of being inferior to 12%). Therefore, the chances that this molecular alteration will have a practical role for patient care in this setting are minimal.

Integrative approaches using multiple types of data, such as more comprehensive analysis of the transcriptome, epigenetic regulators of the genome and modern quantitative proteomics methods, coupled with conceptual bioinformatics and statistical methods that incorporate the intratumor genetic and phenotypic heterogeneity found in cancers, may result in developments that could potentially be translated into clinical benefit for cancer patients[41]. Advances in the understanding of the molecular biology of ER+ breast cancer led to a revolution in this field with the development of a variety of therapeutic agents that are now being used in routine clinical care in patients with metastatic disease such as CDK4/6, PI3K and mTOR inhibitors.[22] However, a clinically useful biomarker to identify primary sensitivity and resistance to endocrine therapy has not yet being developed and remains an unmet need.

CONCLUSION

Growing evidence supports the notion that there are different mechanisms of primary and secondary endocrine resistance. Our study suggests that *ESR1* mutations do not evolve rapidly and do not represent a common mechanism of primary endocrine resistance in the neoadjuvant setting. Therefore, ESR1m should be considered a mechanism of acquired endocrine resistance in the context of advanced disease. Further research should be conducted to identify factors associated with intrinsic resistance to ET.

CONFLICTS OF INTERESTS

Tomás Reinert - Research funding: AstraZeneca. Speaker honoraria: AstraZeneca, Lilly, Novartis, Pfizer, PierreFabre.

Carlos Barrios: Research funding, Speaker honoraria, Advisory role: Novartis, Roche, Pfizer, GSK, Boehringer Ingelhein, Eisai, AstraZeneca, BMS, MSD, Libbs.

All other authors declare no conflict of interest.

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8 – CONSIDERAÇÕES FINAIS

O papel de ESR1m vem sendo estudado em pacientes com câncer de mama avançado após a progressão ao IAs usados para tratamento de doença metastática, usualmente com um padrão de resistência endócrina secundária (ou adquirida). Entretanto, o potencial papel de ESR1m no câncer de mama em estádio inicial e que recebem hormonioterapia (neo)adjuvante não foi adequadamente estudado. O entendimento de mecanismos de resistência primária (ou intrínseca) é de fundamental importância, uma vez que pode estar ligado ao desenvolvimento de biomarcadores e agentes terapêuticos que podem ser incorporados no tratamento com intuito curativo desta doença[28].

A hormonioterapia neoadjuvante vem sendo utilizada com maior frequência não apenas como uma opção terapêutica mas também como uma ferramenta científica para a avaliação do comportamento tumoral em termos de sensibilidade ou resistência à terapia endócrina. A hormonioterapia neoadjuvante permite uma observação *in vivo* da resposta tumoral à deprivação estrogênica dentro de uma "janela de curabilidade". A expressão de Ki67 na peça cirúrgica após o uso de IAs, em conjunto com outros três fatores prognósticos independentes (tamanho tumoral, comprometimento linfonodal e expressão de RE), foi incorporada em uma ferramenta prognóstica chamada PEPI (preoperative endocrine prognostic index) que é um escore que foi validado para distinguir tumores sensíveis ou resistentes à hormonioterapia. Nos estudos clínicos IMPACT e PO24, a taxa de recidiva foi extremamente baixa em pacientes com PEPI escore baixo. Entretanto, um escore PEPI elevado (seja por carga tumoral residual na cirurgia ou expressão alta de Ki67) foi associada com um incremento significativo no risco de recidiva[42]. Portanto, a identificação de tumores com escore PEPI elevado permite não apenas uma personalização do tratamento dos pacientes com maior risco de recidiva, mas também é uma oportunidade para pesquisas translacionais buscando decifrar mecanismos de resistência intrínseca à hormonioterapia utilizada no câncer de mama em estágio inicial.

Nosso estudo demonstra que, diferentemente dos tumores com resistência aos IAs no contexto metastático, mutações no gene *ESR1* não se desenvolvem rapidamente e provavelmente não são um mecanismo comum de resistência primária aos agentes endócrinos no contexto neoadjuvante. Este achado tem implicações no desenvolvimento de linhas de pesquisas translacionais assim como no desenvolvimento de pesquisas clínicas com novos agentes endócrinos para o arsenal terapêutico em oncologia mamária. A descoberta de biomarcadores associados com resistência endócrina intrínseca continua uma necessidade não resolvida e deve ser encarado como uma prioridade para pesquisas futuras.

Existem pesquisas em andamento avaliando uma variedade de potenciais mecanismos de resistência endócrina intrínseca, como defeitos no maquinário de reparo do DNA e interação da via do RE com uma série de alterações moleculares[37], como por exemplo sinalização aberrante da via do FGFR[43].

Nosso estudo possui limitações e reconhecemos que a prevalência encontrada de ESR1m pode estar subestimada uma vez que foi utilizada a metodologia de PCR analisando exclusivamente as mutações mais comumente detectadas de ESR1m, portanto mutações raras em outros códons do gene *ESR1* que potencialmente poderiam ser encontradas com uso de tecnologias de sequenciamento de nova geração não foram avaliadas. Adicionalmente, nós reconhecemos que apesar do imenso desafio de conduzir uma coorte com mais de uma centena de pacientes tratadas como hormonioterapia neoadjuvante, o tamanho amostral relativamente pequeno de 23 tumores com escore PEPI alto possui limitações e realça as dificuldades de estudar resistência endócrina primária.

Entre as qualidades do nosso estudo, nós ressaltamos a condução de uma coorte prospectiva multi-institucional que incluiu 127 pacientes com câncer de mama tratados no contexto de saúde pública e provada com um protocolo padrão de tratamento endócrino. Todas as pacientes foram avaliadas de acordo com diretrizes internacionais de análise anatomo-patológica e imuno-histoquímica. Adicionalmente, utilizamos uma metodologia de biologia molecular validada e pudemos fazer uma comparação pré-planejada do papel de ESR1m em dois contextos (neoadjuvante e metastático) na mesma população utilizando dados publicados por nosso próprio grupo. Apesar do tamanho amostral relativamente pequeno, nós demonstramos que a prevalência de ESR1m em câncer de mama RE+ HER2-negativo resistentes ao uso de IA neoadjuvante é extremamente baixa (0% na nossa amostra e com 95% de chance de ser inferior a 12%). Portanto, as chances que esta alteração molecular venha a ter alguma implicação ou aplicação clínica são mínimas.

9 – PERSPECTIVAS ‘FUTURAS

-

Este estudo foi desenhado para responder à pergunta sobre o papel de mutações no gene ESR1 como mecanismo de resistência endócrina em câncer de mama estádio inicial após tratamento com hormonioterapia neoadjuvante. Concluímos que mutações em ESR1 não são identificadas neste contexto e que seu papel como mecanismo de resistência intrínseca à hormonioterapia não possui implicações clínicas, diferentemente do seu papel em doença metastática. Isso faz com que a pergunta que motivou o desenvolvimento desta pesquisa continua em aberto: quais são os mecanismos de resistência endócrina intrínseca (primária) em pacientes com câncer de mama passível de tratamento com intuito curativo.

Abordagens integrativas utilizando análises compreensivas de genômica e proteômica que capturem a heterogeneidade tumoral do câncer de mama conjuntamente com métodos modernos de análise de dados podem resultar em desenvolvimentos fundamentais com implicações clínicas para benefício dos pacientes. Avanços recentes no entendimento da biologia molecular do câncer de mama RE+ possibilitaram uma revolução na oncologia mamária e o desenvolvimento de agentes que modulam mecanismos de resistência endócrina que foram incorporados na prática clínica atual em doença metastática. Entretanto, em doença inicial os avanços não vêm sendo tão significativos.

Perspectivas futuras incluem manter o interesse no estudo de hormonioterapia neoadjuvante e mecanismos de resistência endócrina em câncer de mama com seguimento da linha de pesquisa nesta área. O foco será buscar a manutenção desta parceria multi-institucional e seguir estudando as valiosas informações epidemiológicas, clínicas e translacionais contidas nesta coorte prospectiva que foi desenvolvida e conduzida com o objetivo de servir de plataforma para uma série de estudos translacionais nesta área. Projetos envolvendo a avaliação de biomarcadores possivelmente associados a resistência endócrina primária estão em desenvolvimento e tem interesse especial no papel do sistema imunológico com avaliação de linfócitos tumorais, alterações moleculares em vias de reparo do DNA e o papel da via do FGFR.

12 – ANEXOS

ANEXO I – STROBE CHECKLIST DO ARTIGO PRINCIPAL

TABELA DE ITENS QUE DEVEM SER DESCRITOS EM ESTUDOS OBSERVACIONAIS, SEGUNDO A DECLARAÇÃO STREIGHTENING THE REPORTING OF OBSERVATIONAL STUDIES IN EPIDEMIOLOGY (VERSÃO EM PORTUGUÊS PUBLICADA POR Malta M et al, Rev Saude Publica 2010, 44(3): 559-65

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ANEXO II – Parecer consubstanciado CEP

UNICAMP - CAMPUS CAMPINAS

PARECER CONSUBSTANCIADO DO CEP

Elaborado pela Instituição Coparticipante

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: ESTUDO PILOTO DA FREQUÊNCIA DA MUTAÇÃO DO GENE ESR1 EM PACIENTES COM CÂNCER DE MAMA LUMINAL A-like TRATADAS COM TERAPIA ENDÓCRINA NEOADJUVANTE

Pesquisador: Tomás Reinert

Área Temática:

Versão: 3

CAAE: 93308518.8.3001.5404

Instituição Proponente: Hospital da Mulher Prof. Dr. José Aristodemo Pinotti - CAISM

Patrocinador Principal: CEPESG - CENTRO DE PESQUISAS DA SERRA GAUCHA LTDA - ME

Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Não

CAMPINAS, 26 de Outubro de 2018

Assinado por:
Renata Maria dos Santos Celeghini
(Coordenador(a))

ANEXO III – Comprovante de submissão do artigo principal para o periódico científico "Breast Cancer Research and Treatment"

BREA - Acknowledgement of Receipt

Breast Cancer Research and Treatment (BREA) <em@editorialmanager.com>
Qui, 14/11/2019 11:28
Para: Tomas Reinert <tomasreinert@hotmail.com>

Dear Dr Reinert,

Thank you for submitting your manuscript, ESR1 mutations are not a common mechanism of endocrine resistance in patients with estrogen-receptor positive breast cancer treated with neoadjuvant aromatase inhibitor therapy, to Breast Cancer Research and Treatment.

During the review process, you can keep track of the status of your manuscript by accessing the journal web site:

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With kind regards,
Springer Journals Editorial Office
Breast Cancer Research and Treatment

ANEXO IV – Artigo secundário publicado no periódico "Journal of Oncology"

Title: Association of ESR1 mutations and visceral metastasis in patients with estrogen receptor-positive advanced breast cancer from Brazil

Tomás Reinert[1,2,7], Guilherme Portela Coelho[3], Jovana Mandelli[3], Edinéia Zimermann[3], Facundo Zaffaroni[4], José Bines[5,6], Carlos Henrique Escosteguy Barrios[7], Marcia Silveira Graudenz[1]

Affiliations

1 – PPG Ciências Médicas, Universidade Federal do Rio Grande do Sul (UFRGS) – Porto Alegre, Brazil

2 – Centro de Pesquisa da Serra Gaúcha (CEPESG) – Caxias do Sul, Brazil

3 – Grupo Diagnose Patologia, Genética e Biologia Molecular - Caxias do Sul, Brazil

4 – FARO STAT Solutions – Porto Alegre, Brazil

5 – Instituto Nacional do Câncer (INCA) – Rio de Janeiro, Brazil

6 – Clínica São Vicente – Rio de Janeiro, Brazil

7 – Latin American Cooperative Oncology Group (LACOG) – Porto Alegre, Brazil

Corresponding author

Tomás Reinert

CEPESG

Rua Vinte de Setembro 2304, 701 – Caxias do Sul, RS, Brazil

tomasreinert@hotmail.com

Article type: Research Article

K**eywords**: breast cancer; endocrine therapy; *ESR1*; ESR1 mutations; aromatase inhibitors;

Abstract

Mutations in the ESR1 gene (ESR1m) are important mechanisms of resistance to endocrine therapy in estrogen receptor-positive advanced breast cancer and have been recognized as a prognostic and predictive biomarker as well as a potential therapeutic target. However, the prevalence of ESR1m in real-world patients has not been adequately described. Therefore, we sought to evaluate the prevalence of ESR1m in metastatic samples from Brazilian patients with estrogen receptor-positive (ER+) advanced breast cancer previously treated with endocrine therapy. The presence of ESR1m was evaluated in formalin-fixed paraffin-embedded (FFPE) breast cancer tissue using real-time quantitative polymerase chain reaction (RT-qPCR). Mutations in codons 380, 537 and 538 of the ESR1 gene were analyzed. Out of 77 breast cancer samples, 11 (14.3%) showed mutations in the ESR1 gene. ESR1m were detected in a variety of organs, and the D538G substitution was the most common mutation. In visceral metastasis, ESR1m was detected in 25% (8/32) of the samples, whereas in non-visceral metastasis an ESR1m was detected in 6.7% (3/45) of the samples. The odds of a sample with visceral metastasis having an ESR1 mutation is 4.66 times the odds of a sample of non-visceral metastasis having an ESR1 mutation (95% CI: 1.13 – 19.27; p-value = 0.0333). Our study indicates that the prevalence of ESR1m in samples from Brazilian patients with metastatic ER+ breast cancer is similar to the described in patients included in clinical trials. We observed an association of ESR1m with visceral metastasis.

Introduction

Estrogen receptor-positive breast cancer is the most common breast cancer subtype. Endocrine therapy (ET), a targeted treatment to the estrogen receptor (ER) pathway is the fundamental initial therapeutic approach in all stages of the disease[1]. Nonetheless, clinical resistance associated with progression of disease remains a significant therapeutic challenge[23]. Mutations of the ESR1 gene, which encodes the ER protein, have been increasingly identified as a mechanism of endocrine resistance[4].

The potential clinical implications of ESR1 mutations (ESR1m) remained underappreciated for more than a decade after its discovery since initial studies focused on primary tumors, where the prevalence of ESR1m is very low[5]. Subsequently, it was demonstrated that breast tumors undergo genomic evolution and ESR1m have been described in 9-40% of patients with advanced ER+ breast cancer resistant to aromatase inhibitors[36478]. ESR1m is a biomarker of worse prognosis and is being evaluated as a predictive biomarker as well as a potential therapeutic target[9].

Despite recent advances in the field, several questions remain unanswered about ESR1m such as the prediction of which tumor will develop this mechanism of resistance. At the same time, the majority of data is derived from patients included in clinical trials, more frequently in developed countries, and little is known about mechanisms of ET resistant in real-world patients, especially in the population from low- to middle- income countries. We aimed here to evaluate the prevalence of ESR1m mutations in metastatic tumor tissues from breast cancer patients from Brazil.

Methods

From the archive of the Pathology Department at a single academic center, we collected formalin-fixed paraffin-embedded (FFPE) tissue specimens from consecutive patients enrolled between 2014 and 2017 with recurrent or metastatic breast cancer previously treated with endocrine therapy. Only tumors of ER-positive HER2-negative metachronous metastasis were selected. All hematoxylin and eosin (H&E) and immuno-histochemistry (IHQ) slides from tumor samples were reexamined by a pathologist who confirmed the diagnosis of metastatic carcinoma and quality (amount of reminiscent neoplastic tissue on paraffin-embedded archived tissue) of each specimen. Additionally, all the lesions were diagnosed as breast metastases by IHQ using one or more of the following markers: GATA3, GCDFP-15 and/or mammaglobin.

In each sample, the tumor area was marked by the pathologist and a cut of approximately 35mg was performed, followed by the extraction of the genetic material (DNA) with the Wizard© Genomic DNA Purification kit (Promega). DNA was quantified using Qubit Fluorometric Quantitation (Thermo Fischer Scientific), and 20ng/µl was the threshold for the analysis of the mutation. The reactions were performed with the equipment 7500 Fast Real-Time PCR System using TaqMan Genotyping Master Mix, primers and TaqMan© probes, from Applied Biosystems (Foster City, CA) following all recommendations of the manufacturer. The analyzed mutations were Y537N, Y537C, Y537S, E380Q, D538G. To detect the presence of the mutation a Taqman© reference probe was used, followed by analysis in the 7500 Software v2.06 (Thermo Fischer Scientific)

A sample size of 81 patients was calculated with an estimated prevalence of 30%, a desired precision of estimate of 0.1 and a confidence level of 0.95. The primary endpoint was the prevalence of ESR1m. The secondary endpoint was the association of ESR1m and site of metastasis (visceral versus non-visceral). Data were analyzed using descriptive statistics. Logistic regression was applied in order to estimate the OR (odds ratio) and 95% Confidence Interval (95% CI). A p-value less or equal to 0.05 was deemed to be significant. This project was reviewed and approved at the IRB institutional review board (Ethical Committee).

Results

Seventy-seven samples were included in the analysis. Of the initial 81 selected samples, 4 were removed from the analysis due to an insufficient amount of extracted DNA (all from bone metastasis). The prevalence of ESR mutation was 14.3% (11 samples). ESR1m were detected in metastatic tissues from different organs such as pleura (n=3), liver (n=2), lung (n=2), ovary, lymph node, bone and chest wall. The most frequently detected mutation was the D538G substitution(n=5), followed by mutations in codon 537 (3 Y537N substitutions, 2 Y537C, and 1 Y537S). No mutations in codon 380 were detected. For more information on the molecular biology analysis, see Supplementary Material.

The probability of having an ESR1 mutation was modeled considering the information regarding local of metastasis (see Table 1). In visceral metastasis, ESR1m was detected in 25% (8/32) of the samples, whereas in non-visceral metastasis an ESR1m was detected in 6.7% (3/45) of the samples. Despite the low number of cases with mutation (reflected in the wide CI), the logistic regression showed that the odds of a sample with visceral metastasis having an ESR1

mutation is 4.66 times the odds of a sample of non-visceral metastasis having an ESR1 mutation (95% CI: 1.13 – 19.27; p-value = 0.0333).

Table 1 – Association of ESR1m with the site of metastasis - n (%).

	Visceral Metastasis	Non-Visceral Metastasis	Total
ESR1 Mutation	8 (25.0%)	3 (6.7%)	11 (14.3%)
ESR1 without Mutation	24 (75.0%)	42 (93.3%)	66 (85.7%)
Total	32 (41.6%)	45 (58.4%)	77 (100.0%)

Discussion

Estrogen receptor-positive (ER+) tumors are the most frequent form of breast cancer and responsible for most of the deaths caused by this disease[10]. ET is the mainstay of ER+ breast cancer therapy in all stages of the disease. In the metastatic disease setting, the use of ET agents is associated with clinical benefit in the majority of patients. Nonetheless, disease progression associated with a complexity of mechanisms of resistance remains a significant challenge[10].

ER, a protein encoded by the ESR1 gene, is expressed in the majority of breast cancers. ER expression is one of the defining features in classifying tumor subtype and assigning therapeutic strategies in breast cancer. Translational and clinical research has established the fundamental role of ER and its hormonal ligands in normal mammary gland development, and in the etiology and progression of breast cancer.[11]

Estrogen hormones have genome-wide transcriptional activities that regulate the expression of a network of molecular pathways that are important in various physiological and

pathological processes.[12] Functionally, the ER consists of two transcriptional activation domains: the N-terminal, ligand-independent activation function domain (AF-1), and the C-terminal, ligand-dependent AF-2 domain. The ligand-binding domain (LBD) resides in the C-terminal region, while the DNA-binding and hinge domains are positioned in the central core of the protein.[2] Estrogen binding triggers a number of events resulting in activation of ER and induces conformational changes in the LBD, allowing the estrogen-ER complex to bind to specific DNA sequences while interacting with co-repressor and co-activator proteins to regulate the transcription of estrogen-responsive genes. Breast tumors undergo genomic evolution during therapy, with the development of new alterations that confer resistance to therapy. ESR1 is known to undergo LBD mutations, gene amplification or translocations that are potential mechanisms of resistance to ET.[13] [14] [15]

Physiologically, estrogens promote a balanced activation of liganded and unliganded transcriptional functions of the ER. When ligand-dependent ER signaling is suppresses by either estrogen deficiency or disfunction of the receptor, a strong upregulation of unliganded ER activation and subsequent resistance to endocrine therapies.[16] The absence of estrogen results in a compensatory increase in the activity of the AF1 domain accompanied by a significant increase in the expression levels of both coding and non-coding RNA transcripts[17].

Despite the relatively high frequency of elevated ESR1 copy numbers in breast tumors[18], the clinical relevance of ESR1 gene amplification as a prognostic or predictive biomarker is not clear requires further study[15]. However, mutations in the ESR1 gene have been consistently recognized as an important mechanism of resistance to aromatase inhibitors (AIs), with a prevalence that ranges from 9 to 40%, usually described from liquid biopsies collected from patients mostly included in randomized clinical trials in developed countries. [9] [19] [20]

ESR1m are most commonly missense mutations clustered in codons 537 and 538 of the LBD. Remarkably, the majority of ESR1m localize to just a few amino acids within or near the critical helix 12 region of the ER LBD, where they are likely to be single-allele mutations, as pictured in Figure 1**.** [3]The most prevalent ESR1 point mutations are Y537S and D538G, while several others have been identified at significantly lower frequencies. ESR1m have been consistently associated with inferior outcomes and are being evaluated as predictive biomarkers to help guide therapeutic decisions[21]. At the same time, the development of specific targeted therapies directed to ESR1-mutant clones is an appealing concept with interesting preclinical data already published and promising clinical work in progress[22]. [23]

INSERT FIGURE 1

Our study reports that the prevalence of ESR1m in real-world patients with breast cancer in Brazil is similar to that described in the literature. This finding has implications related to the development of a line of research of mechanisms of ET resistance in the neoadjuvant setting as well as to the design and conduction of clinical trials evaluating new generation selective ER degraders (SERDs) in an ESR1m-enriched cohort of patients. Despite the low number of cases with mutation, our data show a significant association of visceral site of metastasis and ESR1m. Early studies reported ESR1m in tumor samples obtained from different sites, including visceral and non-visceral metastasis, suggesting that these mutations do not display specific organotropism[2425]. Contrastingly, multivariable analyses based on liquid biopsies of patients from the PALOMA3 and SOFEA trials reported that the detection of ESR1m is associated with bone and visceral disease, suggesting that ESR1m are infrequently detected in locoregional recurrences.[2627]. In our study, ESR1m was identified in locoregional and distant metastasis in a variety of visceral (lung, liver, pleura, ovary) and non-visceral sites (bone, chest wall, lymph nodes) indicating that these mutations do not have organotropism and suggesting that this mechanism of ET resistance could be associated with more aggressive disease phenotypes that usually present with hepatic and pleuro-pulmonary metastasis.

The generation of real-world data is an issue with practical implications for global breast cancer research and it remains a challenge, especially in low- to middle-income countries (LMIC). Translating clinical research achievements into global clinical practice is the clear objective. Clinical trials are designed and conducted in a controlled fashion with specific inclusion and exclusion criteria. Nonetheless, the confirmation of patients´ characteristics and outcomes in a more general population remains an integral part of the process. Observational studies have demonstrated significant clinical and epidemiological differences among breast cancer patients compared to patients from developed countries, with a higher proportion of patients with locally-advanced tumors and young patients, especially among the population treated in the public health system.[28]

Nevertheless, the potential differences in the molecular epidemiology of breast tumors in real-world patients from LMIC have not been adequately studied. The prevalence of biomarkers in breast cancer may vary in different regions of the world. A retrospective observational study with more than five thousand breast cancer patients demonstrated that the distribution of molecular subtypes of breast tumors differed according to geographic regions within Brazil and suggested that a variety of characteristics including socioeconomic and

nutritional status as well as the proportion of African ancestry have to be considered to explain this heterogeneity[29]. It is important to understand the molecular characteristics of breast cancer in the Brazilian population in order to develop adequate public health programs and policies as well as to the development of therapeutic strategies and clinical trials. As an example, recently presented real-world data indicate a lower prevalence of PDL-1 expression in non-small cell lung cancer patients in Brazil. The authors suggested that possible explanations for this discrepancy are inadequate sample handling, pre-analytical issues, or epidemiology of the biomarker, all of which may have impacted the results of biomarkers outside clinical trials.[30] The unquestionable impact of breast cancer and the ongoing culture of globalization should be seen as opportunities to tackle critical global cancer research priorities, such as the development of research in LMIC, the encouragement of independent academic research, the improvement of access to clinical trials and the development of international collaborations.

Our study has several limitations including its retrospective nature, relatively low sample size and the low number of ERS1m identified. Additionally, DNA extraction was unsuccessful in four samples of bone metastases, even though successful DNA extraction was achieved in the majority of bone samples (10 out of 14). We recognize that the detected prevalence of ESR1m can be underestimated given the fact that a PCR-based methodology was used and only specific mutations in the most commonly mutated codons were analyzed, therefore cases with mutations in different codons of the ESR1 gene potentially detectable with next-generation sequencing technologies were not identified[3132]. Another potentially important fact that might decrease the prevalence is that many patients in this cohort were treated with AIs in the adjuvant setting whereas recent data suggest that ESR1m is probably more commonly associated with resistance to the AIs used in the metastatic disease setting[8].

This study is one of the first steps in a project of developing a comprehensive line of translational research in breast cancer through a collaboration of independent academic centers in Brazil. The publication of data of molecular biomarkers in real-world patients that are consistent with data from researches with patients treated in clinical trials is essential to allow validation of our methodology and to provide information for the development of translational and clinical research projects.

Conclusion

The prevalence of ESR1m in samples from Brazilian patients with metastatic ER+ breast cancer is similar to the described in patients included in clinical trials. A significant association

among ESR1m and visceral site of metastasis was detected. ESR1m have potential clinical applications in breast cancer as a biomarker and a therapeutic target.

Conflicts of Interests

Tomas Reinert - Research funding: AstraZeneca. Speaker honoraria: AstraZeneca, Novartis, Pfizer, PierreFabre.

Carlos Barrios: Research funding, Speaker honoraria, Advisory role: Novartis, Roche, Pfizer, GSK, Boehringer Ingelhein, Eisai, AstraZeneca, BMS, MSD, Libbs.

All other authors declare no conflict of interest.

Data Availability Statement

The results reported in the manuscript are publicly available and are included in an online file in Supplemental Materials.

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