Universidade Federal do Rio Grande do Sul

Faculdade de Medicina

Programa de Pós-Graduação em Medicina: Ciências Médicas

CARACTERIZAÇÃO DE UM GRUPO DE PACIENTES EM RISCO PARA CÂNCER DE MAMA E OVÁRIO HEREDITÁRIOS QUANTO A PRESENÇA E FREQÜÊNCIA DE REARRANJOS GÊNICOS EM *BRCA.*

Ingrid Petroni Ewald

Orientadora: Profa. Dra. Patricia Ashton Prolla

Tese de Doutorado

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Leonardo da Vinci

RESUMO

O câncer de mama é uma das neoplasias malignas mais comuns que afetam mulheres de todo o mundo. No Brasil, o Estado do Rio Grande do Sul tem índices de incidência e mortalidade por câncer de mama que situam-se entre os maiores do país. Aproximadamente 5-10% dos diagnósticos são causados por mutações germinativas em genes de predisposição entre os quais estão *BRCA1* e *BRCA2*, associados à Síndrome de Câncer de mama e Ovário Hereditários (*Hereditary Breast and Ovarian Cancer Syndrome* ou HBOC, OMIM #114480).A identificação dos casos hereditários de câncer de mama é importante porque indivíduos afetados apresentam risco cumulativo vital muito superior ao da população para o desenvolvimento de câncer, porque familiares de um afetado podem estar igualmente em risco porque há medidas de rastreamento intensivo e intervenções preventivas que podem diminuir significativamente o risco de câncer em portadores de mutação. O diagnóstico molecular da síndrome HBOC é laborioso e caro devido à heterogeneidade molecular da doença. Famílias que apresentam características indicativas de uma síndrome de predisposição ao câncer de mama e ovário hereditários, mas que são negativas para mutações pontuais em *BRCA1/2* vêm sendo testadas para grandes rearranjos visto que essas anormalidades têm sido consideradas como respondendo por, no mínimo, 10% do todos os casos HBOC com mutação identificável, incluindo grandes deleções ou duplicações. Um estudo recente de Portugal, demonstrou que um rearranjo fundador no exon 3 de *BRCA2* ocorre em por 8% das famílias HBOC do Norte do país. Os objetivos deste trabalho incluíram a verificação da freqüência e caracterização de rearranjos gênicos nos genes *BRCA1* e *BRCA2,* incluindo a mutação fundadora c.156_157ins*Alu* no exon 3 de *BRCA2* em famílias brasileiras de

alto risco para a síndrome HBOC. Em um grupo de 145 indivíduos em risco não-relacionados rastreados para a mutação fundadorac.156_157ins*Alu* no exon 3 de *BRCA2* foram encontrados 3 portadores da mutação (prevalência de 2%). Em um grupo de 145 indivíduos de risco não-relacionados rastreados para rearranjos gênicos em *BRCA1* e *BRCA2* pela técnica de MLPA (*multiplex ligation-dependent probe amplification*) foram identificados 4 portadores de mutação germinativa, sendo a mutação em dois deles um rearranjo gênico no gene *BRCA1* (1,4%) envolvendo sequencias *Alu.* Rearranjos gênicos em *BRCA1* e *BRCA2* são responsáveis por uma parcela das mutações em famílias HBOC Brasileiras. O presente estudo, envolvendo uma série grande de famílias com o fenótipo da síndrome HBOC, não identificou novos rearranjos fundadores, no entanto, demonstrou a presença de rearranjos tanto em *BRCA1* quanto em *BRCA2*, reiterando a importância da busca ativa por estas alterações, que dificilmente são identificadas por técnicas convencionais de sequenciamento gênico. A técnica de MLPA associada a um protocolo específico para detecção da mutação fundadora Portuguesa c.156_157ins*Alu* podem ser utilizadas como estratégia inicial de rastreamento de mutações em famílias Brasileiras com a síndrome. Os resultados apresentados aqui, no entanto, indicam que mutações serão identificadas em menos de 10% dos casos utilizando esta estratégia.

PALAVRAS CHAVE: Câncer de mama, genes BRCA, rearranjos gênicos e mutação fundadora.

ABSTRACT

Breast cancer is one of the most common malignancies affecting women worldwide. In Brazil, the State of Rio Grande do Sul has incidence rates and mortality from breast cancer are among the largest in the country. Approximately 5-10% of the cases are caused by germline mutations in predisposing genes including *BRCA1* and *BRCA2* are associated with the syndrome of breast and ovarian cancer Hereditary (Hereditary Breast and Ovarian Cancer Syndrome or HBOC, OMIM # 114480). The identification of inherited cases of breast cancer is important because affected individuals have cumulative risk life much higher than the population for developing cancer because of an affected family may also be at risk because there are measures of intensive screening and preventive interventions that can significantly decrease the risk of cancer in mutation carriers. The molecular diagnosis of HBOC syndrome is laborious and expensive due to the molecular heterogeneity of the disease. Families that have characteristics indicative of a cancer predisposition syndrome of hereditary breast and ovarian cancers, but are negative for mutations in *BRCA1*/*2* have been tested for large rearrangements because these abnormalities have been identified as accounting for at least 10 % of all cases HBOC identifiable mutation, including large deletions or duplications. A recent study from Portugal, the founder showed that a rearrangement in exon 3 of BRCA2 occurs in 8% of HBOC families of the north. The objectives of this work included the verification of the frequency and characterization of gene rearrangements in BRCA1 and BRCA2 genes, including c.156_157insAlu founder mutation in exon 3 of BRCA2 mutations in Brazilian families at high risk for HBOC syndrome. In a group of 145 individuals at risk unrelated traced to c.156_157insAlu founder mutation in exon 3 of 3 found BRCA2 mutation carriers (prevalence 2%). In a group of 145 individuals at risk unrelated screened for gene

rearrangements in BRCA1 and BRCA2 by the technique of MLPA (multiplex ligation-dependent probe amplification) identified four carriers of germline mutation, and two of the mutation in a gene rearrangement in the gene BRCA1 (1.4%) involving Alu sequences. Gene rearrangements in BRCA1 and BRCA2 account for a portion of HBOC mutations in Brazilian families. This study, involving a large series of families with HBOC syndrome phenotype, no new rearrangements identified founders, however, showed the presence of rearrangements in both BRCA1 and BRCA2, reiterating the importance of active search for these changes, which hardly are identified by conventional techniques of gene sequencing. The technique of MLPA protocol associated with a specific mutation detection founder Portuguese c.156_157insAlu strategy can be used as initial screening for mutations in families with Brazilian syndrome. The results presented here, however, indicate mutations that will be identified in less than 10% of the cases using this strategy.

KEYWORDS: Breast cancer, BRCA genes, genomic rearrangements and founder mutations.

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AR	Receptores de Andrógenos
BRCA1	Breast Cancer 1
BRCA2	Breast Cancer 2
CCR	Câncer Colorretal
ER	Hormônios Esteróides
FAP	Polipose Adenomatosa Familiar
HBCC	Câncer Colorretal Hereditário Não Poliposo
HBOC	Hereditary Breast and Ovarian Cancer
INCa	Instituto Nacional do Câncer
RCV	Risco Cumulativo Vital
RS	Rio Grande do Sul
TRH	Terapia de Reposição Hormonal

Sumário

1. INTRODUÇÃO

O Brasil apresenta uma taxa bruta de incidência de câncer crescente. O perfil epidemiológico de tumores no país mostra uma sobreposição entre neoplasias normalmente associadas à pobreza, como câncer de estômago, útero, fígado e cavidade oral, e aquelas comumente relacionadas a países desenvolvidos, como câncer de mama, próstata, pulmão e cólon. Os tipos de câncer mais freqüentes nos países desenvolvidos estão associados principalmente a fatores da dieta, tabaco, falta de exercício físico e exposição a uma ampla gama de fatores de risco decorrentes do processo de industrialização e urbanização, destacando-se entre estes a exposição a substâncias químicas e hormônios (Cocco *et al*., 2002Koifman & Koifman, 2003; Gallo *et al*., 2005., Tório *et al*., 2009). Estudos epidemiológicos indicam aumento de sua ocorrência tanto nos países desenvolvidos quanto nas regiões em desenvolvimento. Nos países ocidentais, encontra-se entre uma das principais causas de morte em mulheres. Em relação aos tumores associados à pobreza, destacam-se como possíveis fatores causais o consumo de álcool e cigarro (para tumores da cavidade oral, laringe e faringe), infecção por HPV para o câncer de cérvix uterina e por *Helicobacter pylor*i para o câncer gástrico (Parkin*et a*l., 1997; Britto, 1997). O câncer de mama apresenta maior incidência e mortalidade em estados considerados economicamente mais desenvolvidos do Brasil, com destaque especial para as regiões Sul e Sudeste.

O câncer de mama (CM) é provavelmente o tipo de câncer mais temido pelas mulheres, devido à sua alta freqüência e, sobretudo, pelos seus efeitos psicológicos, que afetam a percepção de sexualidade e a própria imagem pessoal. O Câncer de

mama é uma doença multifatorial, com uma pequena parcela dos casos, considerada como hereditária, ou seja, determinada principalmente por uma mutação germinativa em gene de predisposição de alta penetrância.

Uma das principais e mais estudadas síndromes de predisposição hereditária relacionadas ao câncer de mama é a Síndrome de Predisposição Hereditária ao Câncer de Mama e Ovário (*Hereditary Breast and Ovarian Cancer Syndrome* ou HBOC, OMIM #114480). Esta síndrome é causada por mutações germinativas nos genes de predisposição *BRCA1* e *BRCA2* que são genes grandes e altamente heterogêneos. O diagnóstico de mutações patogênicas em um indivíduo ou família com o fenótipo HBOC envolve geralmente uma abordagem com múltiplas técnicas laboratoriais, de alta complexidade e custo. Embora mutações fundadoras e "*hot spots*" nos genes *BRCA1* e *BRCA2* tenham sido descritos e possam simplificar o diagnóstico da síndrome, elas parecem ocorrer somente em algumas populações. Com base nessas constatações, este estudo foi proposto para verificar a existência de mutações nos genes *BRCA1* e/ou *BRCA2* em amostras de mulheres em risco da população brasileira que pudessem justificar uma abordagem diagnóstica inicial mais custo eficaz da síndrome HBOC.

2. REVISÃO DA LITERATURA

2.1 Epidemiologia do câncer de mama

O câncer de mama (CM) é segundo tipo mais freqüente em incidência e mortalidade no mundo e o mais comum entre as mulheres, respondendo por 22% dos casos novos a cada ano. O impacto global da doença mais que dobrou nos últimos 30 anos, sendo que em 2010 foram estimados mais de 12 milhões de casos novos no mundo, os quais resultaram em cerca de 7,5 milhões de óbitos (*American Câncer Society 2012*; INCa, 2012; DATASUS, 2011). A doença é particularmente incidente nos Estados Unidos e Norte da Europa, intermediária em freqüência no Sul e Leste da Europa e América do Sul, e menos freqüente na Ásia. Porém, nos países asiáticos (particularmente Japão, Cingapura e em áreas urbanas da China), as taxas brutas de incidência vêm aumentando rapidamente e essa mudança tem sido atribuída ao nível sócio-econômico da região, padrão de desenvolvimento e comportamento reprodutivo (Harris *et al.,* 1996; Instituto Nacional do Câncer 2012).

No Brasil o CM é um importante problema de saúde pública sendo o tumor mais freqüentemente diagnosticado em mulheres de todos os estados brasileiros, e a primeira causa de morte por câncer em mulheres brasileiras de todas as idades. Segundo estimativas do Instituto Nacional do Câncer (INCa) para 2012, serão diagnosticados no Brasil, 52.000 novos casos de CM, o que corresponde a 52 novos casos a cada 100.000 mil mulheres (INCa 2012).

O estado do Rio Grande do Sul (RS) aparece como o segundo estado com maior incidência da doença (65 casos de CM a cada 100.000 mulheres) antecedido e precedido pelos estados do Rio de Janeiro e São Paulo (69 e 48 casos a cada 100

000 mulheres, respectivamente). O RS compreende 7% da área nacional e tem aproximadamente 10 milhões de habitantes (IBGE, 2012). É considerado o estado brasileiro com maior expectativa de vida (73,4 anos), superior à média brasileira de 69,0 anos, e os idosos (indivíduos acima de 60 anos de idade) representam aproximadamente 10% da população. Esse dado epidemiológico tem relevância para a discussão das altas taxas de incidência de CM nesse estado, pois é sabido que a incidência desta neoplasia aumenta com a idade. A população do RS é bastante heterogênea, e quando comparada a de outros estados do país, é considerada uma das populações que recebeu maior número de imigrantes europeus, com menor contribuição de nativos (índios) e africanos (Marrero *et al.,* 2005). Porto Alegre, a capital do Rio Grande do Sul, apresenta uma incidência surpreendentemente alta de câncer de mama, 980 novos casos a cada 100.000 mulheres para o ano de 2012 (INCa, 2012). Possíveis fatores de risco para a doença, mais prevalentes na região Sul do Brasil e que poderiam explicar em parte as alarmantes taxas de incidência incluem: predomínio de indivíduos de origem caucasiana, menor número médio de filhos, gestações iniciadas em idades mais avançadas, melhor nível socioeconômico e maior uso de terapia de reposição hormonal (Harris *et al.*, 1996; Andrea T *et a*l., 2007). No entanto, não existe uma explicação clara e definitiva para a observação das altas taxas de incidência de CM no Sul do Brasil. É mais provável que resulte de múltiplos fatores, incluindo aspectos sociais, culturais, ambientais e genéticos.

Em relação à mortalidade por CM, observou-se um aumento em todas as regiões brasileiras nas décadas de 1980 e 1990, mas o risco de morte nas regiões Sul e Sudeste foi pelo menos duas vezes maior. Um dos fatores determinantes mais importantes para a alta taxa de mortalidade por CM no Brasil é o avançado estágio da doença no momento em que as mulheres são submetidas ao primeiro tratamento. Em geral, 50% dos casos são diagnosticados em estágios avançados (Silveira *et a*l., 2008; Gonçalves *et al.,* 2006; Pinho, Coutinho., 2007).O câncer de mama masculino é uma doença incomum, representando cerca de 1% de todos os cânceres de mama, porém, em algumas síndromes de predisposição hereditária, o risco de ocorrência de CM em homens é muito maior do que o da população geral (Giordano et al., 2005; Carmalt et al., 1998). Em relação à idade, para os homens, a média da idade dos pacientes ao diagnóstico é de 60 a 70 anos na maioria dos estudos, sendo relatado que o câncer de mama em homens tende a ser diagnosticado em idade mais avançada do que em mulheres. Em mulheres mais jovens o CM é menos freqüente, existindo um aumento crescente dos índices de incidência específicos por idade até a menopausa. Mulheres brancas apresentam um índice global maior de incidência do que as mulheres negras, sendo esta diferença significante somente após a menopausa. Melhores condições sócio-econômicas também têm sido associadas a maior risco para desenvolver CM e mulheres solteiras apresentam incidência maior de câncer de mama, quando comparadas com as casadas (Fentiman *et al.,* 2006; Andrea T *et a*l., 2007; Matos *et a*l.,2010). A contribuição de fatores genéticos na origem e desenvolvimento de neoplasias malignas da mama pode ser evidenciada pela ocorrência aumentada de câncer de mama em familiares de indivíduos afetados, pela ocorrência de agregados familiares de câncer de mama e pela ocorrência de síndromes geneticamente determinadas, que conferem aos seus portadores um alto

risco de desenvolvimento de câncer de mama e outros tumores. A identificação de indivíduos em risco é fundamental para a prevenção e detecção precoce da doença. (Offit *et al.,* 1998; Son *et al.,*2012).

2.2 Principais Fatores de Risco para Câncer de Mama

O controle do câncer de mama deve priorizar a prevenção e a detecção precoce, entretanto, a alta freqüência em mulheres de todo mundo motivou o estudo intensivo de fatores de risco etiológicos e de fatores de risco modificáveis, que seriam úteis para a definição de estratégias eficazes e preventivas. Os fatores que podem contribuir para o desenvolvimento de um CM compreendem fatores intrínsecos (de predisposição hereditária ou dependente da constituição hormonal) e fatores externos (ambientais, incluindo agentes físicos, químicos e biológicos) (Veronesi *et al*., 2002). Podem ainda estar baseados em estilos de vida e em fatores associados ao nível de desenvolvimento econômico, político e social. Os principais fatores associados a um risco aumentado de desenvolver câncer de mamasão:

História familiar. Mulheres com história familiar de CM têm um risco aumentado de desenvolver esta neoplasia. Este risco aumenta com o número de familiares em 1º grau afetados, (mãe, irmã ou filha). A análise do histórico familiar revela freqüentemente a existência de outros casos da doença com características particulares. Entre essas características podemos citar a existência de: (a) familiares afetados em duas ou mais gerações sucessivas; (b) dois ou mais familiares de primeiro grau com diagnóstico da doença no período da pré-menopausa; (c) familiares com câncer de mama bilateral e, (d) diagnóstico de CM em familiares do sexo masculino. A ocorrência de pelo menos uma dessas características em uma

família sugere a existência de um componente genético hereditário que predispõe à doença (Colditz et al., 1993; Rosenthal *et al*., 1999). A presença de outros tumores associados ao CM pode oferecer informações adicionais acerca do tipo de síndrome de predisposição ao câncer de mama em questão (p.ex., presença de câncer de ovário associado ao CM leva á suspeita da síndrome HBOC, presença de câncer de cólon associado ao CM leva à suspeita da síndrome de câncer de mama e cólon hereditários, ou HBCC), bem como a presença de câncer de mama e sarcomas, ou Li - Fraumeni (Dawn *et al*., 2008).

Idade. O risco de desenvolver CM aumenta com a idade, sendo que o seu aparecimento é raro antes dos 30 anos de idade, e a média de idade ao diagnóstico é de 64 anos, aumenta a partir dos 35 anos e vai duplicando de 10 em 10 anos até estabilizar por volta dos 80 anos de idade. A correlação entre a idade e aumento da doença não é linear, havendo um pico de elevação da incidência de CM em mulheres jovens, diminuição do pico durante e logo após a menopausa, finalizando em um novo pico de incidência alguns anos após a menopausa (*American Cancer Society 2007;* Hankinson *et al*, 2004; Korde *et al*, 2004).

Riscos Reprodutivos. Os contraceptivos orais assim como a terapia de reposição hormonal (TRH), aumentam o risco de CM, entretanto este efeito diminui consideravelmente após a cessação do seu uso. A composição dos contraceptivos orais (tipo e dose de estrogênio e a presença ou não de progesterona) variou consideravelmente no decorrer dos anos. O uso de TRH está associado particularmente com carcinomas lobulares, invasivos, pequenos e com presença de receptores hormonais. Mulheres que iniciaram o uso de contracepção oral antes dos

20 anos de idade apresentam um risco maior de desenvolver CM (*Collaborative Group on Hormonal Factors in Breast Cancer* 1997; Araújo et al., 2007; Magnusson *et al*, 1999). Uma atividade cíclica ovariana longa, com uma menarca precoce e/ou menopausa tardia, também estão associados a um alto risco de desenvolvimento de CM. Mulheres que alcançam a menarca antes dos 11 anos de idade possuem um risco cerca de 20% maior de desenvolver CM ao longo da vida, em comparação às mulheres que alcançam a menarca com mais de 14 anos de idade. A menopausa tardia (após os 54 anos de idade) também aumenta o risco para CM, provavelmente devido a um maior tempo de exposição hormonal. Entretanto, a magnitude do risco atribuído à menopausa tardia em relação ao CM não foi quantificada (Azzena., *et al* 1994; Robbins e Cotran, 2005).A nuliparidade é considerada um fator de risco para CM, assim como a ocorrência da primeira gestação após os 30 anos de idade. No que diz respeito à paridade, verificou-se que mulheres que tenham tido pelo menos uma gravidez, têm um risco de desenvolver CM 25% menor do que mulheres nulíparas. Esta proteção aumenta com o número de gravidezes, atingindo os 50% em mulheres com cinco ou mais filhos (Layde *et al*, 1989; Ewertz *et al*, 1990;Robbins e Cotran, 2005). Longos períodos de lactação reduzem o risco para CM. Um estudo de metanálise evidenciou que a cada nascimento de um filho e a cada ano de amamentação, o risco relativo para CM diminui em 7% e 4,3%, respectivamente (*Collaborative Group on Hormonal Factors in Breast Cancer 1997;* Layde *et al*, 1989; Ewertz *et al*, 1990;Robbins e Cotran, 2005).

Etnia. A incidência e mortalidade por CM variam consideravelmente entre diferentes grupos étnicos. A incidência é maior entre os eurodescendentes e afro-americanos, intermediária entre hispânicos e ameríndios, e mais baixa entre os asiáticos (Ghaffor

et al., 2003). Mulheres eurodescendentes apresentam maior incidência de CM relativamente às mulheres afro-americanas, no entanto, estas últimas apresentarem maior taxa de mortalidade. Apesar dos modernos métodos de detecção precoce do câncer de mama, de intervenção e das terapias pós-operatórias terem melhorado o prognóstico de pacientes com tumor primário de mama, existe uma profunda disparidade racial que, de fato, tem aumentado nos últimos anos (Butler & Cunningham, 2001; Moorman *et al*., 2001). Com relação à distribuição etária e de ancestralidade dos casos diagnosticados, observa-se que pacientes afrodescendentes são diagnosticadas em faixa relativamente mais jovem, em torno de 56 anos, enquanto que pacientes eurodescendentes têm seu diagnóstico em torno dos 60 anos de idade. Mulheres judias, especialmente Ashkenazi e com história familiar de primeiro grau de CM, apresentam um risco cerca de quatro vezes maior de desenvolver a doença (Egan *et al.,* 1998 ; Smigal *et al*, 2006).

Toxinas ambientais. Alguns estudos sugerem que os contaminantes ambientais, tais como pesticidas organoclorados, poderiam ter efeitos estrogênicos em humanos. O possível efeito de toxinas ambientais no CM está sendo intensamente investigado. Nenhuma substância específica foi definitivamente associada a um risco aumentado até o momento (Robbins e Cotran, 2005).

Estilo de vida. Acredita-se que a obesidade e a ingestão de álcool conferem também risco para desenvolver CM após a menopausa. O risco para mulheres que consomem menos de 60g/dl por dia não é significativamente diferente das abstêmias. Acima desta dose, quanto maior a quantidade de álcool ingerida, maior a chance de vir a ter câncer de mama, especialmente se o uso for contínuo ou muito freqüente. Entretanto, o risco relacionado ao álcool para desenvolvimento do CM é correspondente ao risco

da droga para outros tipos de câncer (Boffetta *et a*l., 2006; Friedenson, 2012). Em relação ao tabaco, não há associação definitiva com risco para CM, embora exista risco maior para mastite e outras patologias benignas da mama em fumantes (Robbins e Cotran, 2005;). A alta ingesta calórica, especialmente de gorduras saturadas, relaciona-se a aumento de risco para câncer de mama, e foi postulado que modificações na dieta, limitando consumo diário de gordura para menos de 15-20% da ingesta, diminuiria o risco para a doença (Nordevang *et al.,* 1992). Outros fatores de risco identificados para câncer de mama incluem, o envelhecimento do indivíduo, a residência em determinadas regiões do mundo, a qualidade de vida devido às condições financeiras da família e a exposição a irradiação, citadas na tabela 1.

Tabela 1. Fatores de risco estabelecidos para câncer de mama

Fatores que influenciam o risco	Risco relativo estimado
Envelhecimento (idade 65 – 69 anos vs. idade 30 – 34 anos)	17
Residência na América do Norte ou Europa (vs. Ásia)	4 – 5
Residência em zonas urbanas	1,5
Renda familiar ou escolaridade maior	1.5
Mãe ou irmã com câncer de mama	2 – 3
Nuliparidade ou idade tardia na primeira gestação >30 anos	2 - 3
Ausência de amamentação	1,5
Idade da menarca (<12 anos *vs*. > 15 anos)	1,5
Idade da menopausa (>55 anos *vs*. 45 anos)	2
Doença proliferativa da mama (confirmada por biópsia)	2 - 4
Densidade mamária elevada	2 – 4
Obesidade (no período pós - menopáusico)	2
Alta estatura	1,5 – 2
Irradiação torácica em altas e moderadas doses	2 – 4

História familiar de câncer de mama	2 – 4
História de câncer primário de endométrio ou ovário	1,5 – 2

Modificado de Brinton & Devesa, 1996 - Diseases of the Breast

Fatores de risco para o desenvolvimento de câncer de mama já foram bem estabelecidos, entretanto as causas exatas da ocorrência deste tumor e a magnitude da contribuição de cada um dos fatores de risco identificáveis para determinado indivíduo são difíceis de definir (Jose *et al*., 2004). Fatores de proteção para câncer de mama também podem ser identificados. A ocorrência desta neoplasia é menos provável em mulheres que tiveram pelo menos um filho antes dos vinte anos, mulheres com história de amamentação por períodos prolongados, retirada dos ovários antes dos 35 anos, prática regular de atividade física e aquelas com dietas pobres em gordura. Embora programas de prevenção da exposição a fatores de risco e de estímulo a hábitos saudáveis tenham sido criados globalmente para diminuir a incidência e mortalidade por CM, o diagnóstico precoce (prevenção secundária), por meio da mamografia ou de exame clínico da mama, ainda parecem ser as medidas de maior impacto na mortalidade (Sociedade Brasileira de Mastologia, 2012; Pischon *et al*, 2008).

2.3 Tipos de câncer de mama

Acredita-se que ainda não existe um modelo que possa definir com precisão os múltiplos eventos envolvidos na carcinogênese mamária, entretanto alguns autores defendem que existem múltiplas vias para o desenvolvimento de CM. Clinicamente, os tumores malignos da mama podem ser divididos genericamente em três formas: esporádicos, familiais e hereditários.

Tumores esporádicos correspondem à grande maioria dos casos, resultam da acumulação de mutações a nível somático, sem que exista qualquer mutação germinativa, são predominantemente associadas à exposição a fatores de risco ambientais. (Kenemans *et al*, 2004; Simpson *et al*, 2005; Ward, 2002). Em sua grande maioria, são tumores que aparecem em mulheres com idade mais avançada, e geralmente não há história familiar importante da doença. Os tumores descritos como familiais descrevem casos associados a algum outro diagnóstico de CM na família. Porém, geralmente não se evidencia um claro padrão de herança autossômica dominante, a idade ao diagnóstico dos casos não é precoce, e não se identificam mutações germinativas de predisposição ao CM. Possivelmente, a ocorrência destes "agregados familiares de CM" está relacionada a uma combinação de fatores genéticos (mutações e/ou polimorfismos em genes de baixa penetrância) e ambientais (exposição ambiental, perfil reprodutivo e/ou social comum a diferentes membros de uma mesma família). Os tumores hereditários da mama decorrem de alterações herdadas (mutações germinativas em genes de predisposição de alta penetrância) que conferem uma maior predisposição ao câncer e correspondem a 5-10% de todos os tumores malignos da mama (de La Chapelle *et al*., 1998; Dawn et al.,2008; Byung *et al*.,2012). Estudos moleculares do carcinoma de mama, baseados na identificação de um perfil de expressão gênica por meio do cDNA microarray, possibilitou definir pelo menos cinco sub-grupos distintos: luminal A, luminal B, HER2 Positivo, basal e normal breast-like. O subtipo luminal A, é o subtipo mais comum, representa cerca de 50-60% do total de casos, o qual o fenótipo é RE positivo e HER2 negativo, foi caracterizado pela alta expressão de genes representados pelas células epiteliais luminais. Este fenótipo está associado a um melhor prognóstico e responde à terapêutica com antiestrogênicos. Já o subtipo luminal B, representa

cerca de 10-20% dos tumores de mama, cujo fenótipo é RE positivo e HER2 positivo, caracterizado por baixa ou moderada expressão de genes expressos por células epiteliais luminais. Este fenótipo está associado a um pior prognóstico, associado à recidiva tumoral, por apresentar possíveis similaridades com os tumores RE negativos. O subtipo HER2 positivo, representa cerca de 15-20% dos tumores de mama,cujo fenótipo é RE negativo e HER2 positivo, é caracterizado pela superexpressão de uma das moléculas da família dos receptores de fator de crescimento epidérmico, o HER2.A amplificação do oncogene HER2 e a superexpressão de sua proteína, é utilizada como um importante biomarcador de prognóstico. Os tumores apresentam boas respostas a drogas que atuam como bloqueadores da atividade de HER2, por exemplo, o anticorpo monoclonal trastuzumab. O subtipo basal-like representa 10-20% de todos os carcinomas mamários, o fenótipo é RE negativo e HER2 negativo, caracterizado pela expressão de vários genes expressos nas células progenitoras ou células basais/mioepiteliais. O fenótipo basal-like é positivo para CK5, CK6, CK14, CK17, receptor do fator de crescimento epidérmico (EGFR), P-caderina e p63, que são proteínas expressas nas células basais/mioepiteliais. Associado a mutações em BRCA1, e a um pior prognóstico, não possui alvo terapêutico definido, conseqüentemente, não responde ao tratamento com drogas antiestrogênicas nem com ao anticorpo monoclonal anti-HER2 (Perou*et al*., 2000; Erole*set al*., 2011; Curtis*et al*., 2012).

Para os portadores de mutação no gene *BRCA2* ainda não há um fenótipo tão estabelecido como para *BRCA1*, são mais semelhantes aos tumores esporádicos. Entretanto, são mais freqüentemente RE positivos, HER2 negativos e ciclina D1 positivos do que os tumores esporádicos (Lakhani *et al*, 1998; Honrado *et al*, 2005; Robson *et al*, 2001; Evans e Howell, 2004; Foulkes, 2006; Campeau *et al*, 2008).

O subtipo normal breast-like responde por cerca de 5-10% de todos os carcinomas mamários. Está associado com o aumento da expressão de genes conhecidos por serem expressos pelo tecido adiposo e por outros tipos de células não epiteliais. Estes tumores também mostraram forte expressão para genes epiteliais basais e baixa expressão para genes do epitélio luminal. Apresentam um prognóstico intermediário entre luminal e basal-like e geralmente não respondem bem à quimioterapia neo-adjuvante (Strehl*et al*., 2011; Curtis*et al*., 2012).

2.4 Câncer de mama hereditário

O câncer da mama hereditário foi inicialmente referenciado pelo cirurgião francês Paul Broca, no século XI. Broca descreveu detalhadamente um grupo de mulheres com câncer de mama na família da sua esposa (madame “Z”), onde 10 de 24 mulheres foram afetadas pela doença. A história familiar de câncer da família da esposa de Broca foi publicada em 1866 (Broca, 1866), sugerindopela primeira vez uma predisposição hereditária ao câncer de mama (Figura 2).Em 1926, o ministro da saúde britânico documentou várias evidências indicando que familiares de primeiro grau de mulheres com CM tinham risco de desenvolver a doença (Benett *et al.,* 1999). Meio século após, Anderson (1976) propôs que mulheres com história de múltiplos familiares de primeiro grau com CM possuíam um risco cumulativo vital (RCV) de desenvolver a doença 47 a 51 vezes maior que o risco da população em geral. O mesmo autor relatou que nessas mulheres, o câncer geralmente se desenvolvia antes da menopausa, era bilateral e parecia estar associado à função ovariana. Centenas de estudos posteriores confirmaram os achados iniciais de uma predisposição aumentada ao CM com base em achados da história familiar (Petrakis *et al*., 1977; Ottmann *et al*., 1983; Kozak *et al.,* 1986; Hauser *et al*., 1992; Eisinger *et a*l., 1998).

Os rápidos avanços em técnicas de biologia molecular nas últimas décadas resultaram na identificação de genes que, quando alterados, aumentam significativamente o risco de desenvolver câncer de mama, câncer de ovário e outros tumores, dentre os quais destacam-se os genes supressores tumorais *BRCA1* e *BRCA2* (Miki *et al.,* 1994; Wooster *et a*l., 1994). Estes genes,participam de etapas centrais da via de reparos de quebras bifilamentares de DNA. Outros genes de predisposição ao CM foram identificados e estão igualmente relacionados às formas hereditárias da doença, associados à outras síndromes com transmissão autossômica dominante, conferindo também um risco aumentado de desenvolver CM, sendo, no entanto, menos freqüentes. : Estes incluem *TP53* (Li & Fraumeni 1969), *CHEK2* (Bell *et al*., 1999; Meijers-Heijboer *et a*l., 2003), *ATM* (Savitsky *et al*., 19), *PTEN* (Eng 1997, Lynch *etal*., 1997) *TWIST1* (El Ghouzzi *et al*., 1997; Howard *et al*., 1997; Rose *et al*., 1997) e *STK11* (Giardiello *et al*., 1987; Hemminki *et al*., 1998; Jenne *et al*., 1998; Campeau *et al*, 2008), A identificação de indivíduos em risco para câncer hereditário é importante por várias razões. Primeiro, porque indivíduos afetados apresentam risco cumulativo vital muito superior ao da população para vários tipos de câncer. Segundo, porque outros familiares de um indivíduo afetado podem estar em risco para o câncer hereditário. Terceiro, porque medidas de rastreamento intensivo e intervenções preventivas (cirurgias profiláticas e quimioprofilaxia) se mostram eficazes em reduzir significativamente o risco de câncer em portadores de mutação (Rebbeck *et al.,* 1999; Hartmann *et al*., 1999; Eisen *et al*., 2000; Hartmann *et al*., 2001; Meijers-Heijboer *et a*l., 2001; Shih & Chatterjee., 2002; Kauff *et al*., 2002; Rebbeck *et al*., 2002; Eisen et al., 2005; Dawn C. Allain., 2008; Lynch *et a*l., 2008; Metcalfe *et a*l., 2008). Atualmente, é possível diagnosticar uma mutação genética de predisposição ao câncer muito antes do diagnóstico de um

tumor. No caso da predisposição hereditária ao CM, que é uma doença de início na vida adulta, o diagnóstico pré-sintomático de um indivíduo afetado tem um enorme potencial para redução do risco de ocorrência de câncer e/ou diagnóstico precoce. Por outro lado, a identificação precisa de um indivíduo não-afetado em uma família de risco permite a sua tranquilização e elimina os gastos e complicações do rastreamento e de intervenções preventivas desnecessárias (Grusenmeyer & Wong, 2007; Meropol & Schulman, 2007;Trepanier *et a*l.,2004;).

A história familiar de câncer em familiares de primeiro grau e a presença de alguns aspectos específicos da história, como presença de câncer de mama bilateral, história familiar de câncer de mama e ovário e câncer de mama em indivíduo do sexo masculino, são indicadores importantes de risco para o câncer de mama hereditário, e em especial da síndrome de câncer de mama e ovário hereditários (HBOC). (Easton et al., 2002; Page et al., 2003; Trepanier et al.,2004; Dawn C. Allain., 2008).

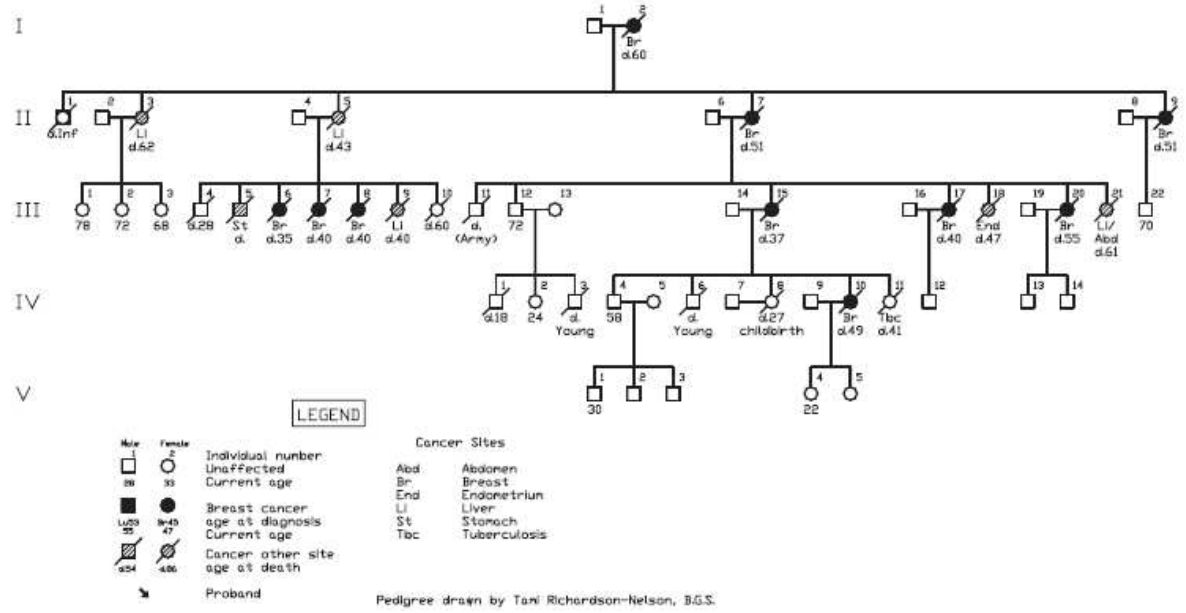

Figura 1. Heredograma da família da esposa de Paul Broca, de acordo com a descrição original deste autor em 1866 (adaptado de Lynch *et al.,* 2008).

2.5 Síndrome de Predisposição ao Câncer de Mama e Ovário (HBOC)

2.5.1 Descrição e riscos associados de câncer

O primeiro gene relacionado à síndrome de predisposição hereditária ao câncer de mama e ovário (HBOC), *BRCA*1 (OMIM #113705), foi identificado em 1994 por Miki*et al*. Esse gene foi mapeado no cromossomo 17q12-23 e sua descoberta foi decorrente de estudos de ligação em membros de famílias com múltiplos casos de câncer de mama e ovário. O segundo gene associado à síndrome, *BRCA*2 (OMIM #600185), está localizado no cromossomo 13q12-13 e, a exemplo de *BRCA*1, também é um gene supressor tumoral (Wooster*et a*l., 1994). Conforme descrito anteriormente, estima-se que cerca de 5-10% de todos os casos de câncer de mama e ovário sejam causados por mutações germinativas em genes autossômicos dominantes de alta penetrância, e, destas, pelo menos 2/3 são mutações em *BRCA1*

e *BRCA*2 (Mikie*t al.*, 1994; Eastone*t a*l., 1995; Woostere*t al.*, 1994; Nathansone*t al.*, 2001; Antoniou*et al.*, 2003; Scott*et a*l., 2003). Acredita-se que *BRCA1* seja responsável por cerca de 45-50% de todos os casos de CM hereditário.A penetrância das mutações germinativas nos genes *BRCA1* e *BRCA2* é incompleta e depende de diferentes fatores, como o tipo de mutação, a população e/ou factores exógenos. Em geral, mulheres portadoras de mutações germinativas no gene *BRCA1* apresentam um risco cumulativo vital de desenvolver CM de 51 a 95% aos 80 anos de idade, enquanto que para o Câncer de Ovário (CO), o risco é de 22 a 65% das portadoras *BRCA1* desenvolvem CO aos 80 anos de idade (Cass*et al.*, 2003; Antoniou*et a*l., 2006; Rische*t a*l., 2006; Bermajo-Perez *et al.,* 2007). Outros tumores que parecem ser mais freqüentes em portadores(as) de mutações em *BRCA*1 incluem câncer de trompa de Falópio, câncer de próstata, tumor de Wilms e câncer de mama masculino (Offit *et al.*, 1998; Thompson & Easton, 2002; Hodgson *et al.*, 2007; Couch*et a*l., 1996; Liede *et* al., 2004; Mai PL *et* al., 2009).

Mutações germinativas em *BRCA2,* estão igualmente associadas ao desenvolvimento de múltiplos tumores. *BRCA2* é responsável por cerca de 30-40% de todos os casos de CM hereditário. O RCV para CM em mulheres portadoras de mutações germinativas nesse gene é similar ao risco de portadoras de mutações germinativas em *BRCA*1 (40-65% até os 80 anos de idade) (Antoniou*et a*l., 2006; Rische*t a*l., 2006; Bermajo-Perez *et al.,* 2007), enquanto que o risco para câncer de ovário é de 15-30% (Cass*et a*l., 2003;). Embora menor que o RCV para câncer de ovário associado a mutações germinativas em *BRCA*1, este risco ainda é 10 vezes maior que o da população em geral (The Breast Cancer Linkage Consortium, 1999). Homens com mutações germinativas em *BRCA*2 têm um RCV significativamente maior que o da população de desenvolver câncer de mama, cerca de 7% até os 70

anos de idade, o que representa um aumento de 80-100 vezes em relação ao risco para a população em geral. Outros tumores identificados em portadores de mutação germinativa em *BRCA*2 incluem: tumores de vias biliares, bexiga, esôfago, pâncreas, próstata, estômago, sistema hematopoiético, cavidade oral, faringe, e melanoma (Offit*et al*., 1998; *The Breast Cancer Linkage Consortiu*m, 1999; Thompson & Easton, 2002; Mai PL *et al*.,2009;Freedman *et* al.,2012).

2.5.2 Diagnóstico clínico

A identificação de Indivíduos portadores de uma mutação germinativa patogênica nos gene *BRCA1* e *BRCA2* é de fundamental importância, tendo em vista, o risco cumulativo elevado de desenvolver CM e CO (Antoniou *et al*, 2003; Kwon *et al*, 2010). Mutações freqüentes ou relacionadas a "*hot-spots*" em *BRCA1* e *BRCA2* não são comumente encontradas. A prevalência e fenótipo da mutação BRCA, varia de acordo com o país e etnia, conseqüentemente, em cada nova família identificada é recomendável pesquisar toda a seqüência codificadora de ambos os genes em busca de uma mutação (Pena *et al,*2006; Son*et al,*2012*)*. Famílias que apresentam um fenótipo sugestivo de HBOC são atendidas em consulta de aconselhamento genético, na qual se explicam as implicações, medidas de vigilância e profilaxia adequadas. Para o diagnóstico clínico de síndrome HBOC, o ideal é documentar detalhadamente a história familiar, mediante registro do heredograma que deve incluir pelo menos três gerações, pelo lado materno e paterno do caso-índice e confirmação de todos os casos de câncer da família. Critérios para o diagnóstico clínico foram desenvolvidos em vários países e geralmente incluem características específicas da síndrome (Tabela 2). Os principais critérios utilizados para diagnóstico clínico da síndrome

HBOC são os critérios de NCCN e ASCO (Statement of the American Society of Clinical Oncology, 1996): que estão descritos detalhadamente no anexo 10.2.4

Tabela 2. Achados do heredograma que sugerem o diagnóstico de predisposição hereditária ao câncer de mama e/ou ovário por mutações em *BRCA1* e *BRCA2*:

Múltiplos casos de câncer de mama diagnosticados antes dos 50 anos de idade;
Câncer de ovário (com história familiar de câncer de mama e/ou ovário);
Câncer de ovário e mama em um mesmo indivíduo;
Câncer de mama bilateral;
Descendência judaica Askenazi e história familiar de câncer de mama e/ou ovário;
Câncer de mama masculino.

Modificado de Ashton-Prolla et al, 2008

Além dos critérios para diagnóstico clínico é possível utilizar modelos de estimativa da probabilidade de existir uma mutação em gene BRCA a partir da história familiar de câncer. Os principais modelos existentes atualmente são os modelos de Couch modificado (Penn II), BRCAPro, e as tabelas de prevalência de mutação do laboratório Myriad (Frank et al., 2002, http://acgh.afcri.upenn.edu,http://www.myriad.com), cuja descrição está detalhada no anexo 10.2.2. Os critérios utilizados para indicar o teste genético para identificação de mutações em gene BRCA variam em diferentes países, mas uma probabilidade mínima de mutação de 10% deve ser considerada. Recomenda-se que o teste deve

sempre ser feito inicialmente em uma pessoa que sabidamente foi diagnosticada com câncer de mama ou câncer de ovário na família em estudo. A identificação de uma mutação germinativa reconhecidamente deletéria em um gene BRCA pode trazer desafios adicionais ao processo de aconselhamento genético, pois não necessariamente um indivíduo portador de mutação desenvolverá câncer. Sabe-se que indivíduos portadores de mutação nos genes *BRCA1* ou *BRCA2* apresentam algumas diferenças em relação aos CM esporádicos, entretanto, apesar das diferentes características clínicopatológicas dos dois grupos, ainda não existem dados suficientes que permitam usar o *status BRCA1/2* como fator de prognóstico nestes indivíduos.

O seguimento dos portadores de mutação nos genes *BRCA1* ou *BRCA2* implica em decisões e medidas de cirurgia profilática ou ainda medidas preventivas não cirúrgicas. A cirurgia profilática tem como objetivo principal, reduzir o risco de desenvolver câncer e consequentemente, sua mortalidade. Entre as principais opções cirúrgicas de redução de risco incluem a mastectomia bilateral profilática, a mastectomia contra-lateral profiláctica e/ou a salpingo-ooforectomia bilateral profilática, cabe salientar que toda cirurgia profilática deve ser considerada em relação a uma série de cuidados e alternativas existentes, que neste caso incluem a vigilância e a quimioprevenção. (Roukos *et al*, 2002). Estudos demonstraram uma redução de 85-95% do risco de desenvolver CM em mulheres de alto risco que realizaram MBP (Meijers-Heijboer *et al*, 2001; Rebbeck *et al*, 2004; Kwon *et* al., 2010).

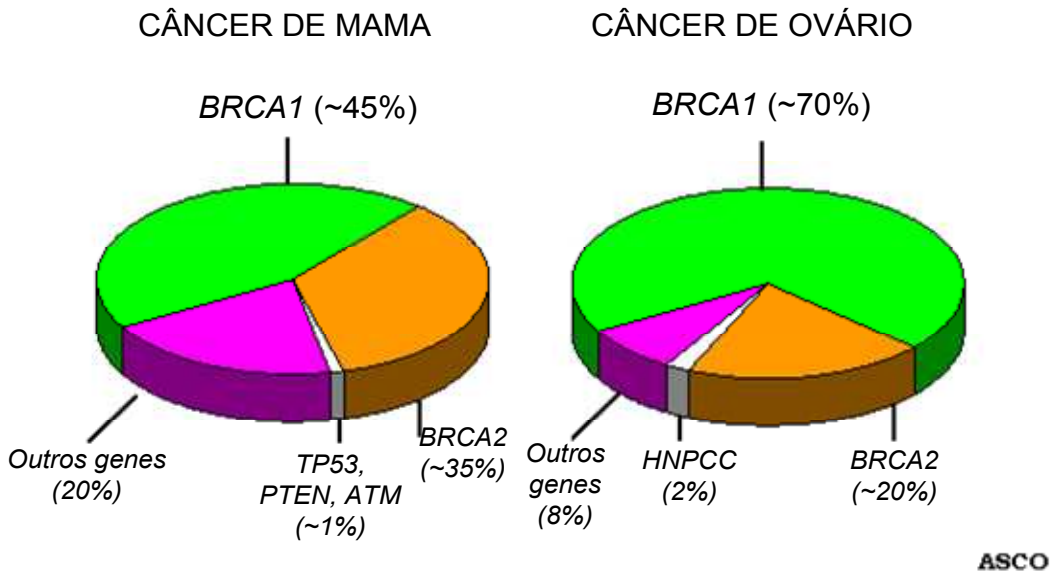

Figura 2. Principais genes associados às formas hereditárias de câncer de mama e câncer de ovário.

2.6 Estrutura dos genes BRCA1 e BRCA2

O gene *BRCA1* localiza-se no braço longo do cromossoma 17 (17q21), contém 24 exons, distribuídos em cerca de 100KB de DNA genômico (Figura 4). Ele codifica uma proteína de 1863 aminoácidos (brca1) que apresenta, na região amino-terminal, um motivo dedo-de-zinco ("Zinc-finger" ou "RING-finger) apresenta importante função na interação de brca1 com diversas proteínas (Boddy *et al*., 1994;Linger*et al*.,2010;). Há considerável variabilidade no processamento do gene decorrente da heterogeneidade das junções intron-exon da região 5' (Fortin *et al.,* 2005). Além do motivo "dedo de zinco" encontram-se, ao longo do exon 11, dois domínios de localização nuclear. A proteína brca1 apresenta uma região de interação à proteína rad51(proteína de reparo) e na região carboxi-terminal, uma concentração de aminoácidos de carga negativa, que formam dois domínios BRCT ("*BRCA* C

Terminus"), envolvidos na manutenção da estabilidade da proteína brca1 (Koonin *et al.,* 1996) bem como na sua interação com outras proteínas (Deng & Brodie, 2000; Wang *et al.,* 2000; Cantor *et al*., 2001; Wang *et al,* 2001; Thompson., 2010). O gene *BRCA2* localiza-se no braço longo do cromossomo 13 (13q12.3), contém 27 exons e codifica uma proteína de 3418 aminoácidos, constituindo uma das maiores moléculas do proteoma humano (figura 3). Na região que compreende o segundo terço da proteína, encontram-se os chamados domínios BRC (*breakpoint cluster region*), estendendo –se ao longo de 1000 aminoácidos. Desta forma, os domínios BRC comunicam-se diretamente através de ligações à Rad51, sendo portanto essenciais para as funções de reparo do DNA desempenhadas pela proteína BRCA2 (Wong *et al*,., 1997;Chen *et al*, 1998; Foulkes.,*et al 2007*).

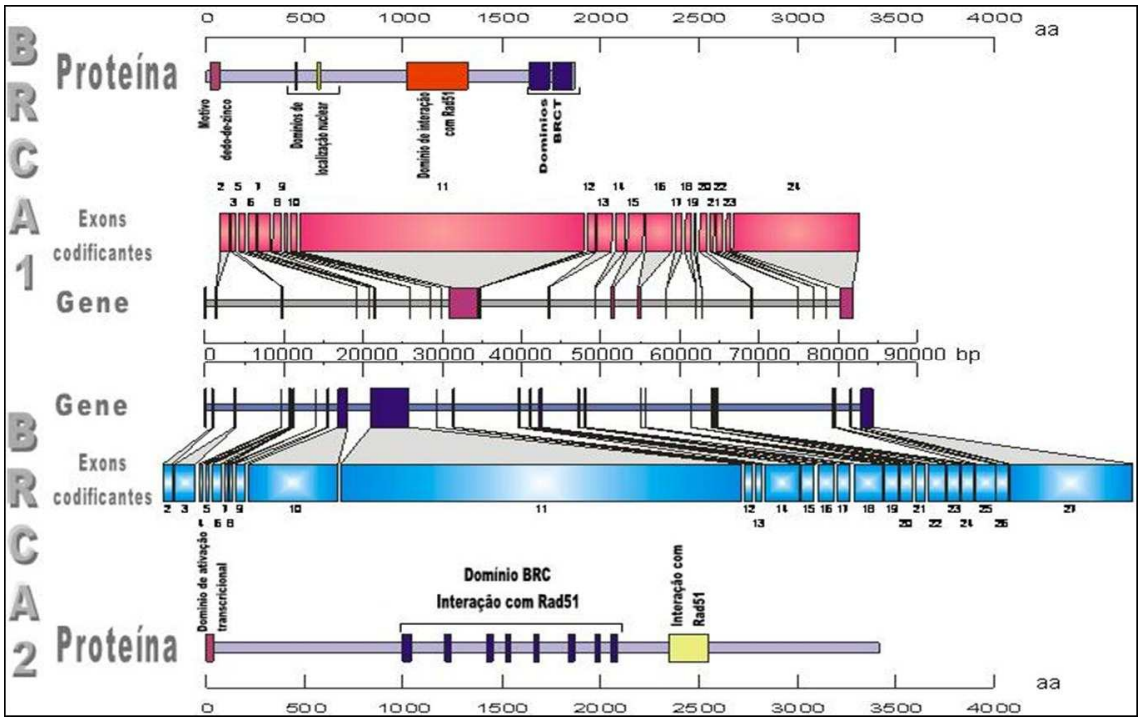

Figura 3. **Representação esquemática dos genes *BRCA1* e *BRCA*2, seus exons codificantes, proteínas e domínios funcionais.**

Nota: A representação gráfica do gene *BRCA*1 foi baseada no Gen Bank (Entry U 14680); a representação gráfica do gene *BRCA*2 foi baseada no Gen Bank (Entry NM 000059). As informações sobre os domínios funcionais de *BRCA*1 foram retiradas de http://www.ebi.uniprot.org (número de

acesso PRO_0000055830); as informações sobre os domínios funcionais de *BRCA*2 foram retiradas de http://www.ebi.uniprot.org (número de acesso PRO_0000064984).

2.7 Funções dos genes BRCA1 e BRCA2

A proteína *BRCA1* predominantemente nuclear desempenha um importantee fundamental papel em vários processos celulares, como reparo do DNA, ativação da transcrição, expressão de outros genes e na resposta a danos celulares (figura 5). Estudos em camundongos deficientes no gene *BRCA*1 têm sido de grande importância para elucidar vários aspectos das suas funções. Camundongos *BRCA1* -/- morrem entre os dias 6.5 e 8.5 pós-implantação por falha de proliferação do blastócito murino (Gowen *etal.*, 1996; Hakem*et a*l., 1996; Liu*etal.*, 1996; Linger et*a*l., 2010). No entanto, a criação de camundongos BRCA-/- TP53-/- retarda, mas não evita, a letalidade embrionária, o que sugeriu que *BRC*A e *TP5*3 pudessem estar em uma mesma rota funcional (Hakem *et al.*, 1997). Camundongos heterozigotos para o gene defeituoso (*BRC*A+/-) têm fertilidade e sobrevida normais e não são predispostos a tumores (Hakem *et a*l., 1996; Liu *et* al., 1996). Já em humanos, a herança de um único alelo defeituoso é suficiente para aumentar a predisposição ao câncer.

A proteína *BRCA1* ativa a expressão de diversos genes, entretanto não sabe-se ao certo, se este efeito é direto ou resulta das funções de reparo e regulação do ciclo celular (Venkitaraman *et a*l., 2001). Bem estabelecida às associações desta proteína na ubiquitinação de outras proteínas, pela presença do domínio RING-*finger* no N-terminal, devidamente envolvido nesta função e também presente na proteína BARD1, interagindo com *BRCA1* nesse local. Acredita-se que esta função também pode ser resultado de *stress* induzido pela replicação do DNA, relacionada com a

função de reparo da proteína BRCA1. A remodelação da cromatina é outro processo em que a proteína BRCA1 está envolvida, ocorrendo em volta de quebras de fita dupla de DNA (*double-strand breaks* - DSBs), facilitando desta forma o reparo do DNA (Morris e Solomon, 2004; Narod e Foulkes, 2004; Foulkes *et a*l., , 2007; Linger et*a*l., 2010). As oito repetições presentes ao longo do exon 11 de *BRCA2* estão envolvidas na interação com a proteína rad51, que atua nos processos de reparo e recombinação.

A proteína apresenta, além desses oito domínios, uma região de ativação transcricional e uma região adicional de interação com rad51 (Bertwistle & Ashworth, 1999). A proteína brca2, juntamente com rad51, está envolvida na manutenção da estabilidade genômica através do seu papel fundamental nos processos de reparo de quebra das duas fitas de DNA por recombinação homóloga (Arnold *et al*., 2006). Estudos realizados com camundongos transgênicos, deficientes para *BRCA2*, revelam que uma perda total do gene acarreta letalidade na maioria dos animais. No entanto, a inativação bialélica em algumas regiões de *BRCA2* pode levar a um fenótipo de anemia, hoje considerado um subtipo da Anemia de Fanconi, doença caracterizada por extrema sensibilidade a agentes causadores de danos cromossômicos, os quais originam quebras cromossômicas e favorecem o desenvolvimento do câncer (Howlett *et a*l., 2002; Arnold *et al*., 2006). A função de ambos os genes (*BRCA1* e *BRCA2*) está relacionada a aspectos centrais ao metabolismo celular, tais como reparo de danos ao DNA, regulação da expressão gênica e controle do ciclo celular (Tutt & Ashworth, 2002; Quaresima *et a*l., 2006). Variações patológicas nesses genes acarretam alterações na transcrição e, especialmente em vias de reparo a danos no DNA, levando ao conseqüente acúmulo de mutações e à instabilidade cromossômica. Dessa forma, mutações em *BRCA1/2*

conferem um alto risco de câncer, mas não ocasionam diretamente o seu surgimento, atuando como genes "cuidadores do genoma" ("*caretakers*"), preservando a estabilidade cromossômica e, quando inativados, facililtando o acúmulo de mutações em múltiplos genes. A natureza das alterações subseqüentes à inativação de *BRCA1/2* é que definirá o destino celular, seja correção do defeito, proliferação celular descontrolada ou apoptose (Cipollini *et al.,* 2004; Rosen *et al*., 2005).

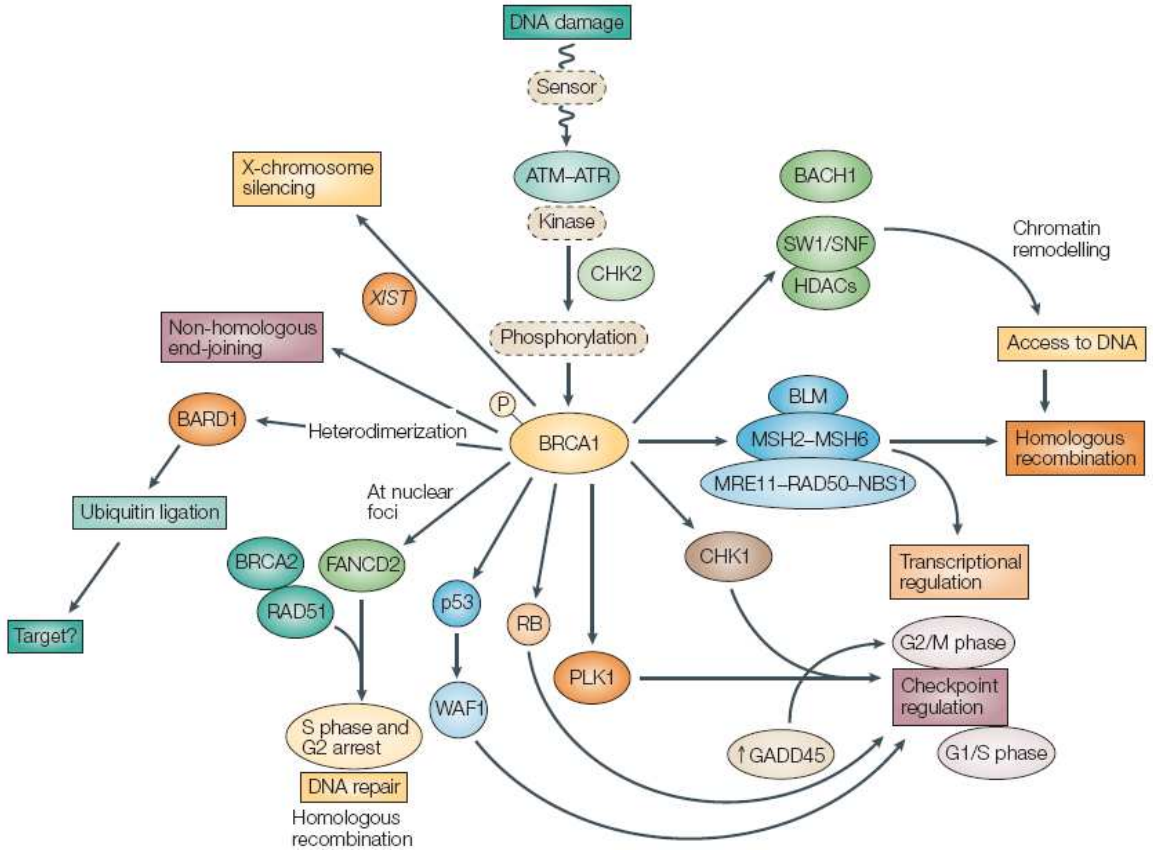

Figura 4. Representação das diferentes interações da proteína BRCA1 e as funções celulares em que está envolvida (Narod e Foulkes, 2004).

Embora não haja grande homologia de seqüência entre *BRCA1* e *BRCA2*, esses genes compartilham diversas similaridades. Dentre elas, destaca-se o fato de que mutações germinativas em ambos os genes predispõe a câncer de mama e de

ovário; ambos codificam proteínas extensas, possuem um primeiro exon não-codificante e um exon central (exon 11) maior que os demais. O exon 11 de *BRCA1* compreende mais de 60% da região codificadora (Bertwistle & Ashworth, 1999). Além disso, ambos os genes são pouco conservados ao longo da escala evolutiva (a proteína brca1 humana apresenta apenas 55,8% de identidade de seqüência com a proteína do camundongo e 74,6% com a do cão), ambos atuam como ativadores transcricionais; ambos se ligam (direta ou indiretamente) a rad51 e possuem um padrão similar de regulação do ciclo celular (Lakhani *et al.,* 1998; Abkevich *et al*., 2004).As proteínas brca1 e brca2 têm um papel central no reparo de quebras bifilamentares de DNA (Figura 5).

Em resposta a agentes causadores deste tipo de dano ao DNA (agentes exógenos ou endógenos, tais como irradiação ionizante e espécies reativas de oxigênio), dois sistemas principais de reparo são acionados na célula: recombinação homóloga e não homóloga, mediados pelos produtos dos genes *ATM* e *ATR*. O reparo por recombinação homóloga geralmente ocorre nas fases S e G2 pode ser ainda subdividido nos em dois mecanismos distintos: conversão gênica (conservativo) e anelamento de fita simples (não-conservativo). Os produtos dos genes *BRCA1* e *BRCA2* estão envolvidos diretamente com o mecanismo conservativo de conversão gênica. Essa função pode ser demonstrada *in vivo* nos tumores resultantes de mutações germinativas em um destes genes que apresentam uma disfunção no reparo de quebras bifilamentares por recombinação homóloga e, conseqüentemente, são hiperssensíveis a drogas que ocasionam esse tipo de dano ao DNA (p.ex. mitomicina C e os análogos da platina) (Lord *et al*., 2006). Em relação à especificidade tecidual de *BRCA1/2* (os tumores resultantes de mutações de perda de função nesses genes ocorrem principalmente em órgãos hormônio-responsivos como

mama, ovário, útero e próstata), acredita-se que esta esteja relacionada à sua função na co-regulação da transcrição de certos genes em órgãos-alvo específicos.

Diversas evidências indicam que *BRCA1* se liga a fatores de transcrição seqüência-específicos e, dessa forma, estimula ou inibe a transcrição. Com base nesses achados, supõe-se que brca1 interaja diretamente com os receptores de hormônios esteróides (ER), inibindo-os e, ao mesmo tempo, estimulando os receptores de andrógenos (AR). Assim, se alguma alteração deletéria ocorrer em *BRCA1/2*, sua deficiência promove, por exemplo, um excessivo crescimento dos tecidos epiteliais da mama, devido à falta de regulação negativa dos receptores estrogênicos (Rosen *et al*., 2005; Mai*et al*., 2009;).

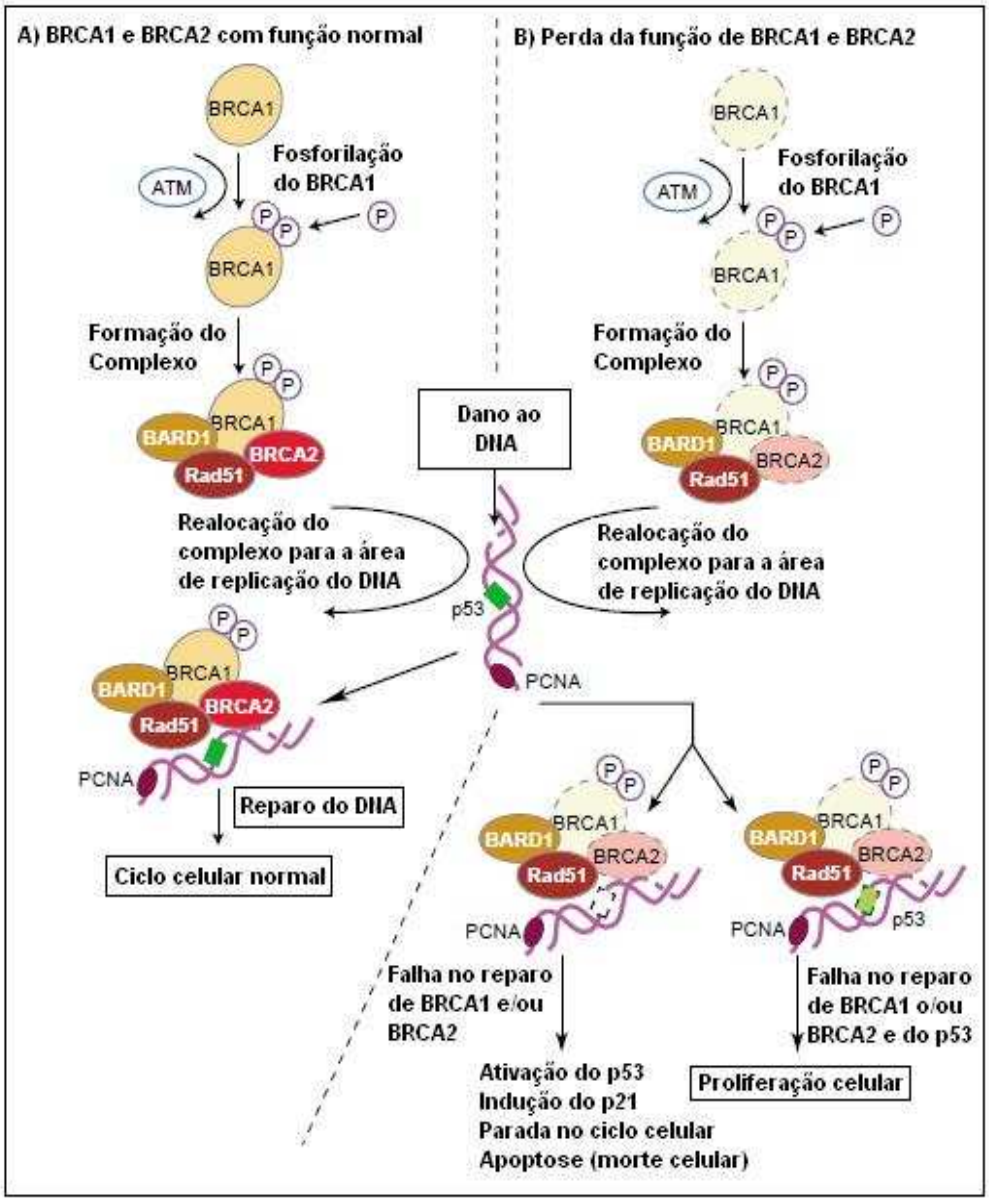

Figura 5. O papel dos genes *BRCA1* e *BRCA2* no reparo do DNA. A= Complexo de reparo normal formado pelas proteínas BRCA1, BRCA2, BARD1 e RAD51, atuando no reparo eficaz do dano ao DNA. B= Perda da função do complexo de reparo por alterações de perda de função de *BRCA1* e/ou *BRCA2* (indicado por linhas pontilhadas) levando a incapacidade de reparo do DNA danificado. Quando o gene *TP53* está com sua função normal, a célula é induzida ao processo de apoptose, mas se o gene *TP*53 esta com sua função comprometida, ocorre a proliferação celular. (Modificado de Arnold e Gioggins, 2001).

2.8 Mutações e Rearranjos Gênicos em BRCA1 e BRCA2:

A identificação de mutações patogênicas em famílias com critérios HBOC é determinante no seu seguimento clínico, permitindo desta forma o diagnóstico pré-

sintomático (Domchek e Weber, 2008). A patogenicidade de muitas mutações nestes genes pode ser facilmente interpretada pela natureza das mesmas, como é o caso de grandes deleções exônicas e mutações do tipo *frameshift,* que resultam em umaalteração na grade de leitura do RNA. Existem também, mutações denominadas *nonsense,* que consistem basicamente na troca de um nucleotídeo, convertendo o códon em que ocorre a troca, em um stop códon (tabela 3). Estes tipos de mutações originam transcritos instáveis ou resultam na perda de domínios funcionais importantes da proteína, denominadas como patogênicas, ou seja, mutações que causam perda na função da proteína (Chenevix-Trench *et al*, 2006). Mutações pontuais incluindo inserções ou deleções de uma ou duas bases ou substituições de um aminoácido são os principais tipos de mutações deletérias encontradas em genes BRCA de famílias com a síndrome HBOC. Recentemente, além de mutações pontuais, grandes "rearranjos gênicos" em *BRCA*1 e *BRCA*2 vêm sendo identificados e associados ao fenótipo HBOC. Estas mutações são encontradas principalmente em *BRCA*1, e variam de 0,5 a 23,8 kb e incluem deleções ou duplicações de grandes fragmentos do gene, incluindo um ou mais exons, Payne *et al*., 2000; descreveram a primeira grande deleção genômica que resulta na omissão do éxon 3 no mRNA do *BRCA1* maduro. Essa deleção mantém a seqüência de leitura (ORF- Open Reading Frame), porém cria um códon de parada prematuro.

Uma grande proporção de rearranjos gênicos tem sido observado entre as mutações deletérias de *BRCA1* nos Países Baixos, devido à ocorrência de mutações fundadoras que representam 27 – 36% dos diagnósticos (Preisler *et al.,* 2006; Hogervorst *et al*., 2003). Uma proporção similar de rearranjos entre as mutações deletérias de *BRCA1* foi descrita em uma pequena população ao norte da Itália (Montagna *et al*., 2003). Em contraste, famílias Dinamarquesas e Finlandesas com a

síndrome HBOC apresentam uma freqüência bem menor de rearranjos entre as mutações patogênicas do gene, indicando uma menor importância em termos de freqüência para este tipo de mutação nos países nórdicos (Thomassen et al., 2006; Lahti-Domenici *et al.,* 2001). A freqüencia de rearranjos entre as famílias HBOC de diversos países é bastante variável, tendo sido relatadas as freqüências de 6%,12% e 5,7% na República Tcheca, Estados Unidos da América e Alemanha. No Canadá, este tipo de mutação não parece ser prevalente (Vasickova et al., 2007; Hartmann *et al.*, 2004, Walsh *et al*., 2006; Moisan *et al.*, 2006; Sluiter et al., 2010).

Tabela 3. Mutações nos genes *BRCA1* e *BRCA2* descritas no HGMD (The Human Genome Mutation Database).

Tipo de mutação germinativa	Número	
	BRCA1 N (%)	BRCA2 N (%)
De ponto, não-sinônima/Stop códon	381 (34%)	270 (32%)
Em sítios de splicing	95 (9%)	58 (7%)
Pequenas deleções	349 (31%)	341 (40%)
Pequenas inserções	120 (11%)	126 (15%)
In/dels	16 (1%)	14 (2%)
Grandes deleções	116 (10%)	24 (3%)
Grandes Inserções/duplicações	22 (2%)	8 (1%)
Rearranjos complexos	15 (1%)	6 (1%)
Total	1114 (100%)	847 (100%)

Disponível em: http://www.hgmd.org (acessado em 10/05/12)

Acredita-se que a maioria dos rearranjos detectadosem *BRCA1* esteja relacionada a eventos de recombinação desigual entre elementos *Alu,* seqüências repetitivas de DNA que estão associadas a inserções, deleções, recombinações e alterações na expressão gênica (Batzer *et al.,* 2002). Possuem a denominação *Alu* porque a maioria dos seus membros é clivada por uma endonuclease de restrição bacteriana denominada *Alu I.* No total existem cerca de 500.000 membros da família Alu no genoma humano, e estima–se que constituam cerca de 3% do DNA humano.

Seqüências *Alu* correspondem a aproximadamente 41,5% das seqüências intrônicas de *BRCA1* (Gad S *et al.,* 2001;Sharifah *et al.,2010*Sluiter et al., 2010).

Poucos rearranjos têm sido descritos em *BRCA2*, o que pode ser explicado pelo fato que as seqüências intrônicas deste gene contem menor número de repetições *Alu* que *BRCA1.* Aparentemente, rearranjos neste gene são mais comuns em famílias HBOC com câncer de mama masculino (Woodward *et al*., 2006; Tounier *et al*, 2004; Ritva *et al*., 2006; Sara *et al*, 2007; Sharifah *et al, 2010*). Para uma revisão detalhada sobre rearranjos gênicos em genes BRCA, consultar o manuscrito 1.

A maioria das mutações patogências em *BRCA1* e *BRCA2* são "privadas" (descritas em uma única família) ou então, comuns a somente poucas famílias. Ocasionalmente, se observa uma alta freqüência de determinada mutação em uma população específica, geralmente decorrente de efeito fundador. O efeito fundador pode ser definido como a ocorrência com alta frequência de uma ou mais mutações especificas em dada população, originárias de um ancestral comum. O efeito fundador é caracterizado como um fenômeno de evolução. Acontece quando uma população em um ambiente isolado é invadida por apenas alguns indivíduos com determinado genótipo, que então se multiplicam rapidamente. De acordo com o princípio do fundador, criado em 1954, caso um grupo muito pequeno de indivíduos de uma população a deixe e vá fundar uma nova população, a freqüência gênica e a variância da nova população poderão ser diferentes, em comparação à população original, dependendo de vários fatores como, por exemplo, da freqüência de casamentos consangüíneos entre os membros da nova população (Burns &Bottino 1991).

Diversas mutações fundadoras foram identificadas em indivíduos de diferentes origens, incluindo judeus Ashkenazi (185delAG e 5382insC em *BRCA1* e 6174delT

em *BRCA2)*, canadenses (C4446T e 2953del3+C), noruegueses (1675delA e 1135insA), alemães (2804delAA), gregos (5382insC e G1738R), islandeses (999del5), espanhóis (330AG, 6857_6858del, e 9254_9258del), e suecos (3171ins5). (Tabela 4). Além disso, estudos atuais têm identificado um grande número de novas mutações do tipo rearranjos gênicos nos genes *BRCA1* e *BRCA2* em famílias HBOC de origem Portuguesa: a inserção *Alu* no exon 3 do gene *BRCA2* (c.156_157insAlu), identificada primeiramente em uma paciente portuguesa que vive na Bélgica, tem sido considerada o rearranjo mais freqüente em pacientes portugueses com HBOC, e foi considerada como uma mutação fundadora portuguesa (Machado *et al.*, 2007; ; Teugels *et al*., 2005.,Peixoto *et al*., 2009).

Tabela 4. Mutações fundadoras em genes *BRCA*

População	Gene *BRCA1*	Gene *BRCA2*	Referência
Africana	1832del5, 5296del114		Gao et al, 1997
Judaica Ashkenazi	185delAG, 5382insC	6174delT	Simard et al, 1994
Britânica	4184del4	6503delT	Neuhausen et al, 1996
Finlandesa		L2776X	Vehmanen et al, 1997
Portuguesa		c.156_157ins*Alu*	Teugels et al, 2005
Francesa	5149del4	9254del5, A2951T	Stoppa Lyonnet et al 1997
Húngara	5282insC		Ramus et al, 1997
Islandesa	999del5		Thorlacius et al, 1996
Italiana	1499insA		Montagna et al, 1996
Holandesa	2804delAA		Peelan et al, 1996
Norueguesa	1136insA		Andersen et al, 1996
Sueca	3166insTGAGA, 2595delA, 1201del11, G563X		Johannson et al, 1996
Russa	5382insC, 4153delA	4486delG	Hakansson et al, 1997

2.9 Diagnósticode Síndrome HBOC

A população Brasileira é extremamente heterogênea, tendo recebido imigrantes dos mais diferentes grupos étnicos ao longo dos séculos, mutações comuns ou fundadoras não são particularmente freqüentes no País, exceto em algumas regiões específicas, em que efeito fundador pode ser demonstrado para

alguns subgrupos da população. (Severini *et al*., 1999; Ribeiro *et al*., 2001; Jardim *et al*., 2001). Consequentemente, na grande maioria das famílias HBOC é recomendável avaliar toda a região codificadora de ambos genes *BRCA,* um processo laborioso, complexo e caro (Pena*,* 2006). Essa dificuldade resulta do tamanho desses genes e da extensa heterogeneidade molecular observada na doença. Atualmente, duas estratégias principais são utilizadas para identificação de mutações germinativas na seqüência codificadora dos genes *BRCA*: a) seqüenciamento de todos os exons codificadores de ambos os genes e posterior análise comparativa da seqüência obtida com uma seqüência de referência (p.ex. GenBank), ou b) rastreamento de mutações utilizando uma de diversas técnicas: *Denaturing High Performance Liquid Chromatography* – DHPLC (Oefner & Underhill, 1995, Underhill *et a*l., 1997),*Single Strand Conformation Polymorphis*m – SSCP (Markoff*et al*., 1997),*Protein Truncation Tes*t – PTT (Hogervorst*et a*l., 1995) ou*Denaturing Gradient Gel Electrophoresi*s – DGGE (Fodde & Losekoot, 1994) ,*Comparative genomic hybridization on microarrays - array-CGH (Staafet al.,2008),*com posterior sequenciamento dos exons com padrão variante identificado no rastreamento inicial.

Abordagens com menor custo e mais eficazes, em relação ao seqüenciamento de toda a região codificadora de ambos genes e à alta prevalência de certas mutações em alguns grupos étnicos, vem sendo frequentemente adotadas, como é o caso da mutação fundadora portuguesa c.156_157insAlu. Para a detecção da mutação, utilizam-se dois PCRS independentes, um para a amplificação do exon 3 e outro específico para a inserção *Alu*. Não sendo encontrada a mutação, se procede então ao teste de mutações ao longo de toda a seqüência codificadora de ambos os genes.

Mais recentemente, a análise de rearranjos gênicos, utilizando técnicas como *Multiplex Ligation Probe-dependent Amplification* – MLPA (Schouten*et al.*, 2002; Hogervorst*et a*l., 2003), *Long-Range Polymerase Chain Reaction* –PCR de longo alcance (Payne*et al.*, 2000) ou*Southern Blotting* (Southern *et al*., 1974), vem sendo realizado em alguns países, especialmente em casos onde os métodos tradicionais geralmente não são capazes de detectar alterações.

O seguimento dos portadores de mutação nos genes *BRCA1/BRCA2* inclui medidas de cirurgia profilática ou de medidas preventivas não cirúrgicas. A cirurgia profilática tem como objetivo reduzir o risco de mortalidade e de câncer nestes portadores. As opções cirúrgicas de redução de risco incluem a mastectomia bilateral profilática (MBP), a mastectomia contra-lateral profilática (MCLP, para doentes já diagnosticados com CM), e/ou a salpingo-ooforectomia bilateral profilática (SOBP) (Roukos e Briasoulis, 2007., Son *et al*.,2012; Kwon *et al*.,2010). Estudos demonstraram uma redução de 85% do risco de desenvolver CM em mulheres de alto risco que realizaram MBP (Meijers-Heijboer *et al*, 2001; Rebbeck *et al*, 2004). Posteriormente, Rebbeck *et al* (2004) verificaram uma redução de risco de aproximadamente 95% em mulheres que realizaram previamente ou concomitantemente SOBP, tornando estas as medidas mais eficazes para mulheres portadoras de mutação nos genes *BRCA1* ou *BRCA2*. Entretanto, as alternativas de prevenção não cirúrgicas, podem incluir a vigilância e a quimioprevenção, assim como intervalos de realização para exames de rastreamento menores do que um ano, devido ao rápido desenvolvimento do Câncer de mama nas portadoras de mutação.

3. JUSTIFICATIVA E OBJETIVOS

3.1 JUSTIFICATIVA

Considerando:

a) A alta incidência do câncer de mama no Brasil,

b) A importância de diagnosticar indivíduos com predisposição hereditária ao câncer de mama pelo potencial de prevenção do câncer nestes indivíduos e seus familiares,

c) o alto custo e complexidade do diagnóstico molecular dos principais genes associados ao câncer de mama hereditário, se justifica um estudo para verificar se existem mutações do tipo rearranjo gênico nos genes *BRCA* entre mulheres Brasileiras com o fenótipo da síndrome HBOC e qual a sua frequência. Da mesma forma, se justifica a busca por uma estratégia simplificada e de menor custo para identificação de mutações patogênicas nos genes *BRCA* como abordagem inicial de identificação de mutações

3.2 Objetivo Geral

Verificar a freqüência de rearranjos gênicos em *BRCA1/2* e a freqüência de uma mutação fundadora Portuguesa de *BRCA2* em uma amostra de indivíduos Brasileiros com diagnóstico clínico de síndrome de predisposição hereditária ao câncer de mama e ovário.

3.2.2 Objetivos Específicos

1. Verificar a frequência de rearranjos gênicos nos genes *BRCA1* e *BRCA2* identificados por MLPA (*Multiplex Ligation-dependent Probe Amplification* – MLPA) em uma estratégia de primeira abordagem de rastreamento molecular.
2. Verificar a freqüência mutação fundadora c.156_157ins*Alu* no exon 3 de *BRCA2* em indivíduos brasileiros
3. Caracterizar famílias com rearranjos de *BRCA1* e *BRCA2* em seus aspectos clínicos (história familiar, tipos de tumores presentes na família, idade ao diagnóstico) e estabelecer correlações genótipo-fenótipo quando possível.

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5. MANUSCRITO 1: Genomic rearrangements in BRCA1 and BRCA2: A literature review

Genomic rearrangements in *BRCA1* and *BRCA2*: A literature review

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Abstract

Women with mutations in the breast cancer genes *BRCA1* or *BRCA2* have an increased lifetime risk of developing breast, ovarian and other *BRCA*-associated cancers. However, the number of detected germline mutations in families with hereditary breast and ovarian cancer (HBOC) syndrome is lower than expected based upon genetic linkage data. Undetected deleterious mutations in the *BRCA* genes in some high-risk families are due to the presence of intragenic rearrangements such as deletions, duplications or insertions that span whole exons. This article reviews the molecular aspects of *BRCA1* and *BRCA2* rearrangements and their frequency among different populations. An overview of the techniques used to screen for large rearrangements in *BRCA1* and *BRCA2* is also presented. The detection of rearrangements in *BRCA* genes, especially *BRCA1*, offers a promising outlook for mutation screening in clinical practice, particularly in HBOC families that test negative for a germline mutation assessed by traditional methods.

Key words: BRCA1, BRCA2, breast cancer, genomic rearrangements, MLPA.

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Introduction

The precise identification of germline *BRCA1* and *BRCA2* mutations is a major concern for geneticists counseling families with a high risk of breast and ovarian cancers. The most frequent mutations encountered in these genes are deletions or insertions of a few bases or single-base substitutions that result in premature stop codons (Perrin-Vidoz *et al.*, 2002; Narod and Foulkes, 2004). Such point mutations occur throughout the coding sequence of both genes and account for 10%-50% of the germline mutations encountered in hereditary breast and ovarian cancer (HBOC) families, depending on the inclusion criteria used (Agata *et al.*, 2005; Vasickova *et al.*, 2007).

The observed frequencies of *BRCA1* mutations are lower than predicted by linkage analysis, with pathogenic variations in the coding region or splice sites of the gene being found in approximately two-thirds of *BRCA1*-linked families. This finding suggests that other dominant genes (Ford *et al.*, 1998; Armour *et al.*, 2002) and/or low penetrance alleles, such as the 1100delC mutation in *CHEK2*, may be associated with the HBOC phenotype (Puget *et al.*, 1999; Nevanlinna and Barker, 2006). Indeed, breast and ovarian cancers have been associated with germline mutations in other genes that are involved in the maintenance of genomic integrity, such as *TP53, PTEN, ATM, NBS1, RAD50, BRIP1* and *PALB2*. Inherited breast cancer is currently considered a highly heterogeneous genetic disease with respect to both the *loci* and alleles involved (Walsh *et al.*, 2006; Walsh and King, 2007).

Large genomic rearrangements have recently been identified in HBOC families and account for a small but still significant proportion of cases in several populations. These mutations are usually pathogenic because deletions or insertions of large genomic sequences within a coding

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region result in out-of-frame translation and usually lead to a mutant peptide of abnormal structure and/or function (Preisler-Adams *et al.*, 2006). These mutations may be overlooked by most of the available screening and diagnostic PCR-based methods that use qualitative rather than quantitative methods and do not detect partial or complete exon losses or gains (Armour *et al.*, 2002). Large genomic rearrangements of *BRCA1* may account for up to one-third of all disease-causing mutations in various populations, while large genomic rearrangements in *BRCA2* are less frequently observed (Hansen *et al.*, 2009).

Frequency of Large Rearrangements

As shown in Table 1, the frequency of large genomic rearrangements varies considerably among populations. Among HBOC families, the highest proportion of *BRCA1* rearrangements has been observed in northern Italy, where large genomic deletions account for approximately one-third of the pathogenic *BRCA1* mutations (Montagna *et al.*, 2003) and the overall prevalence of rearrangements in the families studied is 23%. In the Netherlands, rearrangements also represent a high proportion of all deleterious mutations in *BRCA1* (27%-36% of all germline mutations in the gene) and are attributable to founder mutations (Petrij-Bosch *et al.*, 1997; Hogervorst *et al.*, 2003). In contrast, western Danish families with HBOC have a *BRCA1* rearrangement prevalence of 3.8% (Thomassen *et al.*, 2006). Another study done in Finland failed to detect any rearrangements among 82 families with moderate or high risk for HBOC (Lahti-Domenici *et al.*, 2001). The latter two studies indicate a lower frequency of genomic rearrangements in Nordic countries. Finally, a study in Canada found no evidence of *BRCA1* or *BRCA2* genomic rearrangements in high-risk French-Canadian breast/ovarian cancer families (Moisan *et al.*, 2006).

This wide range in the prevalence of rearrangements is most likely related to the different genetic backgrounds of the populations studied, although the heterogeneity of the clinical inclusion criteria used for HBOC in each study may also have influenced the results. Furthermore, the prevalence of rearrangements will be different in samples that include only *BRCA* mutation-negative individuals by sequencing compared to those that include previously untested individuals at risk for HBOC. More recent studies have encountered an intragenic rearrangement prevalence of 6% and 12%, respectively, in high-risk patients in families from the Czech Republic and the United States of America who were negative for *BRCA1/2* point mutations by sequencing (Walsh *et al.*, 2006; Vasickova *et al.*, 2007). In Germany, the prevalence of *BRCA1* rearrangements is lower, ranging from 1 in 59 (1.7%) to 1 in 17.5 (5.7%) among high-risk families who are mutation-negative by sequencing (Hofmann *et al.*, 2003; Hartmann *et al.*, 2004; Preisler-Adams *et al.*, 2006).

Only a few studies have examined the prevalence of *BRCA2* rearrangements in larger sets of high-risk patients. In a report from Australia, large genomic rearrangements in *BRCA2* were identified in 2% of 149 high-risk families that tested negative for *BRCA1* and *BRCA2* point mutations (Woodward *et al.*, 2005). Agata *et al.* (2005) found a similar frequency (2.5%) of *BRCA2* rearrangements among 121 highly selected Italian families. In a recent study of Portuguese HBOC families, a single founder *BRCA2* rearrangement (c.156_157insAlu) was identified in 8% of the families studied and is the most frequent *BRCA2* rearrangement described to date (Machado *et al.*, 2007).

Molecular Pathology of *BRCA1* Rearrangements

Several *BRCA1* germline rearrangements with well characterized breakpoints have been reported (Mazoyer, 2005). These rearrangements are scattered throughout the gene and although most of them are deletions, duplications, triplications or combined deletion/insertion events have also been described. The *BRCA1* gene characteristically has an extremely high density of intronic *Alu* repeats and a duplicated promoter region containing a *BRCA1* pseudogene that most likely account for the occurrence of "*hot spots*" that favor unequal homologous recombination events (Smith *et al.*, 1996; Puget *et al.*, 2002). Currently, 45 different large genomic rearrangements have been characterized worldwide, including deletions and duplications of one or more exons (Table 1).

Alu sequences

The human genome contains up to 1 million copies of interspersed *Alu* elements (approximately one *Alu* repeat for every 5 kb) that apparently mediate chromosomal rearrangements and homologous recombination events, resulting in translocations, duplications, inversions or deletions (Kolomietz *et al.*, 2002; Tancredi *et al.*, 2004). These sequences are named *Alu* because most of the members of this family of repeats are cleaved by the bacterial restriction endonuclease *Alu I.* Members of the *Alu* family show significant homology but do not have identical sequences. Around 500,000 members of the *Alu* family have been identified and it is estimated that together they comprise 3% of the human genome. Approximately 41.5% of the intronic sequences of *BRCA1* consist of *Alu* elements (Figure 1) that range in size from 0.5 kb to 23.8 kb and are located throughout the entire gene (Montagna *et al.*, 1999).

Alu sequences have often been regarded as genomic instability factors because they are responsible for recombinational "*hot spots*" in certain genes and are frequently involved in exon shuffling during meiosis as a result of non-homologous recombination. These sequences may also act as regulatory factors in transcription, with structural roles (as "physical separators" of protein-protein

Table 1 - Frequency of *BRCA1* and *BRCA2* genomic rearrangements among different populations.

Country	Gene studied	Prev *BRCA*	Prevalence	Proportion*	Rearrangements described	Reference
Australia	*BRCA1/2*	Yes	2%	-	*BRCA1*: Del. ex 3, ex 5, ex 21-23 *BRCA2*: Del. ex 1-2, ex 14-16	Woodward *et al.* (2005)
Canada	*BRCA1/2*	Yes	0%	0%	None	Moisan *et al.* (2006)
Czech Republic	*BRCA1*	Yes	6%	-	Del. ex 1A/1B-2, ex 5-14, ex 11-12, ex 18-19, ex 20, ex 21-22	Vasickova *et al.* (2007)
Denmark	*BRCA1/2*	Yes	1.3%	3.8%	*BRCA1:* Del. ex 3-16, ex 13-15	Thomassen *et al.* (2006)
Finland	*BRCA1/2*	Yes	0%	0%	None	Lahti-Domenici *et al.* (2001)
Germany	*BRCA1/2*	Yes Yes Yes	1.7-5.7%	8%	*BRCA1*: Del. ex 1A/1B-2, ex 5, ex 5-7, ex 17; Dupl. exon 13.	Hofmann *et al.* (2003), Hartmann *et al.* (2004), Preisler-Adams *et al.* (2006)
Italy	*BRCA1*	Yes	23%	40%	Del. ex 1A/1B-2, ex 9-19, ex 18-19, ex 20	Montagna *et al.* (2003)
Italy	*BRCA2*	Yes	2.5%	-	Del. ex 17-18, ex 8-11, ex 20	Agata *et al.* (2005)
Netherlands	*BRCA1*	Yes	7-9.1%	27%-36%	Del. ex 8, ex 13, ex 20-22, ex 22; Dupl. ex 13, ex 21-23; Tripl. ex 17-19	Petrij-Bosch *et al.* (1997), Hogervorst *et al.* (2003)
Poland	*BRCA1/2*	Yes	4.7%	4.5%	*BRCA1:* Del. ex 1A/1B-2, ex 17-19	Ratajska *et al.* (2008)
Portugal	*BRCA1*	Yes	9.6%	-	Del. ex 1-22, ex 8-13, ex 15-16; Dupl. ex 3-8, ex 18-20	Casilli *et al.* (2002)
Portugal	*BRCA1/2*	Yes	1.1%	6.7%	*BRCA1:* Del. ex 11-15	Peixoto *et al.* (2006)
Portugal	*BRCA2*	No	8%	-	Dupl. exon 3	Machado *et al.* (2007)
Portugal	*BRCA1/2*	Yes	1.1%	6.7%	*BRCA1:* Del. ex 11-15	Peixoto *et al.* (2006)
Singapore	*BRCA1/2*	Yes	3%	14.3%	*BRCA1:* Del. ex 13-15; Dupl. ex 13 *BRCA2:* Dupl. ex 4-11	Lim *et al.* (2007)
Spain	*BRCA2*	Yes	1.5%	-	Del. ex 2, ex 10-12, ex 15-16; Dupl. ex 20	Gutierrez-Enriquez *et al.* (2007)
USA	*BRCA1*	Yes	12.7%	-	Del. ex 14-20, ex 22, ex 13; Dupl. ex 13	Hendrickson *et al.* (2005)
USA	*BRCA1/2*	Yes	12%	-	*BRCA1:* Del. ex 1A/1B-2, ex 3, ex 8-9, ex 17, ex 20; Dupl. ex 13, among others	Walsh *et al.* (2006)
USA -Hispanic community	*BRCA1*	Yes	3.8%	-	Del. ex 9-12	Weitzel *et al.* (2007)

Prev *BRCA*: previously *BRCA*-negative patients by sequencing; Prevalence: prevalence of rearrangements in the families studied; Proportion: proportion of rearrangements in relation to all mutations.
(*) In most of the studies, *BRCA* point mutations were not excluded. Del. = deletion, Dupl. = duplication, ex = exon, and Tripl. = triplication.

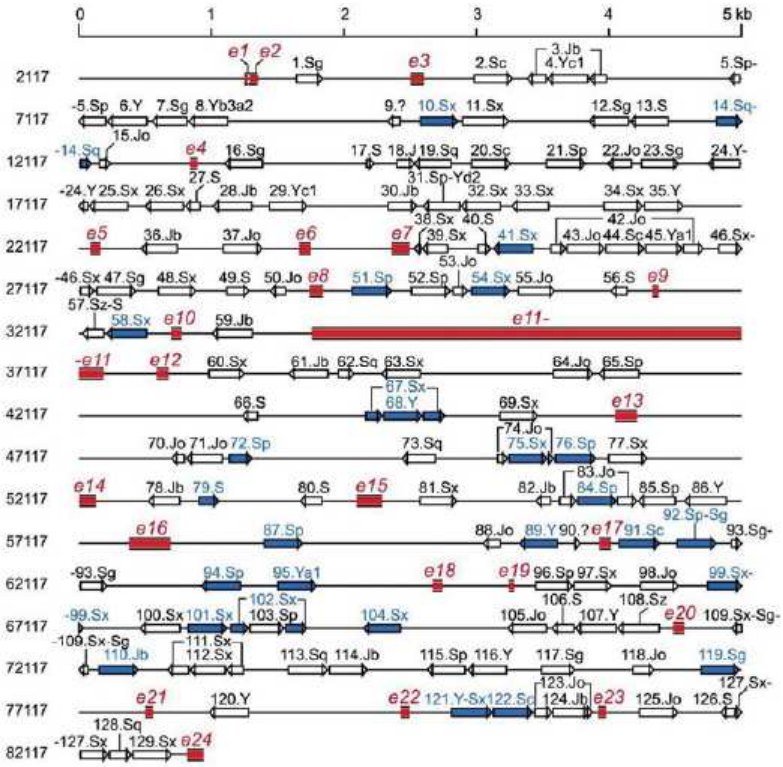

Figure 1 - *Alu* elements in *BRCA1* (reproduced from Pavlicek *et al.* 2004, by permission of Oxford University Press). Exons are depicted as red rectangles and *Alu* sequences as arrows. *Alu* elements known to be involved in human exonic deletions and/or duplications are shown in blue.

interactions during chromosome condensation in cellular division) and functional roles (in alternative "splicing" or as a connection between transcription factors) being proposed.

The two most prevalent sub-classes of repetitive elements in the *Alu* family are LINEs (Long Interspersed Elements) and SINEs (Short Interspersed Elements). LINEs span 6-8 kb and represent ~21% of the total human genome DNA, whereas SINEs, which are derived from RNA polymerase transcripts, are shorter (100-300 bp) and represent ~13% of the human genome. LINEs and SINEs are mobile elements that move via reverse transcription (Gad *et al.*, 2001).

The complete genomic sequence of *BRCA1* was published by Smith *et al.* (1996), who identified 138 individual *Alu* elements within this gene. Rearrangements are less common in the *BRCA2* gene, probably because of a lower frequency of *Alu* sequences (17%). In most of the well characterized rearrangements described in the literature, there is good evidence for the involvement of *Alu* repeat elements in the recombination event. For example, the *BRCA1* exon 5-7 deletion described in German families results from a non-allelic homologous recombination between *AluSx* in intron 3 and *AluSc* in intron 7. Both *Alu* repeats share a homologous region of 15 bp at the crossover site. (Preisler-Adams *et al.*, 2006)

Non-functional pseudogenes

Another important cause of unequal recombination within the coding region of certain genes is the presence of non-functional pseudogenes with high sequence homology to at least parts of the functional gene. Pseudogenes are usually non-functional "relatives" of known genes that have lost their protein-coding ability or are no longer expressed in the cell (Vanin, 1985).

Puget *et al.* (2002) were the first to report this mutational mechanism for the *BRCA1* gene. In two families with HBOC, these authors showed that the first exons of the gene were replaced by those of the *BRCA1* pseudogene, ψ*BRCA1*. This pseudogene had previously been shown to lie ~30 kb upstream of *BRCA1* (Barker *et al.*, 1996; Brown *et al.*, 1996). The presence of a duplication containing most of *BRCA1* exons 1 and 2 and the identification of two different recombination events involving homologous regions located in the *BRCA1* gene and ψ*BRCA1*, respectively, led the authors to postulate that these regions were strong "*hot spots*" for recombination. The mutant alleles identified in

the study harbored a chimeric gene that consisted of ψ*BRCA1* exons 1A, 1B, and 2 fused to *BRCA1* exons 3-24. This chimeric gene lacked both the *BRCA1* promoter and translation initiation codon and was therefore non-functional (Hofmann *et al.*, 2003).

Tandemly arranged short sequence repeats

Gross chromosomal deletions and/or insertions may also be mediated by tandemly arranged short sequence repeats. Highly repetitive nonconding human DNA often occurs in arrays (or blocks) of tandem repeats of sequences which may be simple (1-10 nucleotides) or moderately complex (tens to hundreds of nucleotides). Individual arrays can occur at a few or many different chromosomal locations. Satellite DNA, which constitutes most of the heterochromatic regions of the genome and is particularly noticeable in the vicinity of centromeres, consists of very large arrays of tandemly repeated DNA. Short repeats may cause slipped mispairing during replication, resulting in deletions or duplications of varying sizes. Recombination involving tandemly arranged short sequence repeats underlies the 244 bp deletion in *BRCA1* exon 5 described in German HBOC families (Preisler-Adams *et al.*, 2006).

BRCA2 Rearrangements

Only a few studies have investigated the presence and frequencies of deleterious *BRCA2* rearrangements, and most of these were either done on a relatively small number of families or used cumbersome mutation detection methods of variable sensitivity (Agata *et al.*, 2005).

Until recently, only two genomic rearrangements had been identified in six studies that analyzed hereditary breast cancer patients or primary breast tumors among diverse European populations (Peelen *et al.*, 2000; Lahti-Domenici *et al.*, 2001; Chin *et al.*, 2001; Wang *et al.*, 2001; Gad *et al.*, 2002; Bunyan *et al.*, 2004). The greatly reduced incidence of large genomic alterations that affect *BRCA2* compared to *BRCA1* most likely reflects differences in the density of *Alu* repeat sequences at the two *loci,* and these initial studies were not very supportive of the inclusion of this type of analysis in routine mutation testing of HBOC families (Preisler-Adams *et al.*, 2006).

To date, 16 *BRCA2* germline rearrangements have been reported. More recent studies have reported the frequent occurrence of large genomic *BRCA2* rearrangements in male breast cancer families. Woodward *et al.* (2005) reported three *BRCA2* rearrangements in 25 families with at least one male breast cancer, but no *BRCA2* rearrangements in 114 families without male breast cancer, and Tournier *et al.* (2004) described three *BRCA2* rearrangements in 39 French families with at least one case of male cancer. These findings indicate that large genomic rearrangements in *BRCA2* are more frequent in families with male breast cancer.

Another recent study done in Portugal described a common *BRCA2* rearrangement involving an *Alu* element, c.156_157ins*Alu* in exon 3, in 17 (8%) of 210 HBOC families (Machado *et al.*, 2007).

Methods for Detecting Rearrangements

Classic methods for mutation detection (such as sequencing) are usually unable to identify large genomic rearrangements. Consequently, several alternative methods have been developed for the analysis of structural genomic abnormalities. These methods, which are designed to target either one or a few specific *loci*, or to scan the whole genome, include Southern blotting, long-range PCR, fluorescent *in situ* hybridization (FISH), quantitative multiplex PCR of short fluorescent fragments (QMPSF), protein truncation test (PTT), comparative genomic hybridization (CGH), real-time or quantitative PCR (RT-PCR or qPCR) and multiplex ligation-dependent probe amplification (MLPA). Although each of these methods has potential advantages and limitations, there have been very few large-scale comparative analyses of these techniques. A brief summary of the most common detection methods is provided below.

Southern blotting

Southern blotting is the transfer of DNA fragments from an electrophoretic gel to a membrane support that results in immobilization of the fragments on the membrane and in a semipermanent reproduction of the banding pattern of the gel. This technique can be used to detect changes in copy number (deletions and duplications) when samples are run in parallel (concomitantly) with an internal standard. In addition, large rearrangements may also be detected by a size shift in the blotted DNA fragments. Although frequently used in the past, this method has lost popularity as a routine diagnostic procedure since it is laborious, time consuming, requires large amounts of high-molecular weight DNA and its interpretation may be hampered by false-negative results (Unger *et al.*, 2000; Brown, 2001; De Lellis *et al.*, 2007).

Long-range PCR

Long-range PCR uses a mixture of two thermostable DNA polymerases (proofreading and non-proofreading), thereby increasing the product size to 35 kb. The method has been useful for identifying specific large aberrations, including intragenic deletions, insertions, duplications and chromosomal breakpoints in several disorders. Long-range PCR was originally designed to detect changes in gene copy number rather than translocations or inversions, requires small amounts of DNA and is excellent for locus-specific identification of known rearrangements. These features make it ideal for diagnostic purposes. However, this technique is limited by its low throughput and is unable to provide a genome-wide view of rearrangements, which

therefore restricts its usefulness to the analysis of a specific genomic region delimited by the primers that are used (Vasickova *et al.*, 2007; Morozova and Marra, 2008).

Fluorescent *in situ* hybridization (FISH)

FISH is based on the hybridization of fluorescent probes to metaphase or interphase nuclei followed by analysis with a fluorescence microscope. FISH can detect variations in copy number (deletions and duplications), translocations and inversions. Copy number is assessed by microscopic visualization. The most commonly used conventional *in situ* hybridization protocol in cancer research is dual-color FISH. This method involves labeling centromeres and the DNA region of interest with different colors and estimating the probe copy number from the ratio of the centromeric to noncentromeric signal. Dual-color FISH is used to detect chromosomal gains or losses (aneuploidy), intrachromosomal insertions, deletions, inversions, amplifications and chromosomal translocations. The advantages of FISH include the ability to analyze single cells, applicability to a wide range of substrates, including fixed samples (such as paraffin-embedded tissue), and relative simplicity of use. The method cannot provide a genome-wide assessment of DNA rearrangements, with the exception of gross chromosomal aberrations detected by multifluor-based techniques, and is thus of limited value for genome-wide identification of smaller-scale chromosomal aberrations (De Lellis *et al.*, 2007; Morozova and Marra, 2008).

Quantitative multiplex PCR of short fluorescent fragments (QMPSF)

QMPSF is a sensitive method for the detection of genomic deletions or duplications based on the simultaneous amplification of short genomic fragments using dye-labelled primers under quantitative conditions. The PCR products are analyzed on a sequencing platform used in the fragment analysis mode and the peak height and area are proportional to the quantity of template present for each target sequence. In this setting, the height or area of peaks corresponding to the loss of one allele will be half that of normal samples, whereas a gain of one allele will result in a 50% increase. This method is rapid and sensitive and has been used to screen for *BRCA1* rearrangements (Casilli *et al.*, 2002; Bastard *et al.*, 2007; Weitzel *et al.*, 2007). However, it is not easily implemented in a routine mutation analysis laboratory and requires a fair amount of previous experience.

Protein truncation test (PTT)

The PTT method is a straightforward approach to screen for biologically relevant gene mutations. The method is based on the size analysis of products resulting from transcription and translation *in vitro*. Proteins of lower mass than the expected full-length protein represent translation products derived from truncating frameshift or nonsense mutations in the analyzed gene. Mutation detection may be limited by the size and location of the rearrangement in relation to the primers used in the assay. In addition, because of the low sensitivity of conventional PTT, mutations can be detected only in samples that harbor a relatively high number of mutated gene copies (Peelen *et al.*, 2000; Hauss and Müller, 2007).

Comparative genomic hybridization (CGH)

CGH (also known as chromosomal microarray analysis or CMA) is a molecular-cytogenetic method that has been used to analyze variations in copy number (gains or losses) of DNA from patients and/or tumor cells. The method is based on the hybridization of fluorescently labeled tumor DNA and normal DNA to normal human metaphase preparations. Using epifluorescence microscopy and quantitative image analysis, regional differences in the fluorescence ratio of gains/losses *vs.* control DNA can be detected and used to identify abnormal regions in the genome. CGH does not identify structural chromosomal aberrations such as balanced reciprocal translocations or inversions since they do not change the copy number. Although CGH is a complex technique that requires significant previous experience in cytogenetics and a specific set-up in terms of infra-structure, it is an efficient method for genome-wide screening of rearrangements (Rouleau *et al.*, 2007).

Real time polymerase chain reaction (qPCR)

Real time PCR, also known as quantitative real time polymerase chain reaction (qPCR), is a polymerase chain reaction-based technique used to amplify and simultaneously quantify a target DNA molecule. qPCR allows the detection and quantification (as absolute number of copies or relative amount when normalized to DNA input or additional normalizing genes) of a specific sequence in a DNA sample. The procedure follows the general principle of PCR, the key difference being that the amplified DNA is quantified as it accumulates in the reaction in *real time* after each amplification cycle. Two common methods of quantification are the use of fluorescent dyes that intercalate with double-stranded DNA, and modified DNA oligonucleotide probes that fluoresce when hybridized with a complementary DNA. Although this method is rapid and does not require a large amount of starting material, it has a limited throughput. It is not suitable for the detection of translocations or inversions or for genome-wide screening of rearrangements (Barrois *et al.*, 2004; Morozova and Marra, 2008).

Multiplex ligation-dependent probe amplification (MLPA)

MLPA is a multiplex PCR method developed to detect abnormal copy numbers of different genomic DNA sequences. Each MLPA probe consists of two oligonu-

cleotides that can be ligated to each other when hybridized to a target sequence. All ligated probes have identical sequences at their 5' and 3' ends, permitting simultaneous amplification in a PCR containing only one primer pair. One of the two oligonucleotides of each MLPA probe has a common sequence used for PCR amplification at the 5' end and a target-specific sequence at the 3' end. The 5' region of the second oligonucleotide of each probe is designed to hybridise to the target sequence immediately adjacent to the first oligonucleotide and its 3' region has a common sequence used for PCR amplification and a "stuffer" sequence with different a specific length. Each probe gives rise to an amplification product of unique size, due to the variation in the stuffer sequence length. Because only ligated probes will be exponentially amplified during the subsequent PCR reactions the number of probe ligation products is a measure for the number of target sequences in the sample. The amplification products of different sizes are separated using capillary electrophoresis (Schouten *et al.*, 2002). Nevertheless, MLPA has certain drawbacks, including false-negative scores when probes are designed outside the region of interest, *i.e.*, outside the region involved in the rearrangement. MLPA is primarily used as a screening tool to identify rearrangements, and the precise location of the deletion or duplication breakpoints in the usually very large intronic or affected flanking regions must be refined by sequencing (Staaf *et al.*, 2008). In addition, in rare cases, MLPA may give a false-positive result for a deletion due to occurrence of a point mutation within the sequence of MLPA probe hibridisation (Gomez *et al.*, 2009). However, compared to most other techniques, MLPA is an inexpensive, sensitive, relatively simple, and high-throughput method (Hogervorst *et al.*, 2003; Dunnen and White, 2006; Ratajska *et al.*, 2008). The use of MLPA has facilitated the screening of genomic rearrangements in *BRCA1* (Montagna *et al.*, 2003; Hartmann *et al.*, 2004) and *BRCA2* (Woodward *et al.*, 2005).

Conclusion

Point mutations in the *BRCA* genes are the most common deleterious mutations encountered in HBOC families, and full gene sequencing and other PCR-based methods remain the gold standard for initial mutation identification. However, rearrangements in these genes have been described in a significant proportion of HBOC families, and are responsible for up to one-third of the identifiable *BRCA* mutations in certain populations. Consequently, in HBOC families that test negative for *BRCA* point mutations by conventional approaches, screening for large gene rearrangements in *BRCA1* and probably also *BRCA2* should be strongly considered. A suggested flowchart for investigation in these families is presented in Figure 2. The availability of relatively inexpensive and technically straightfor-

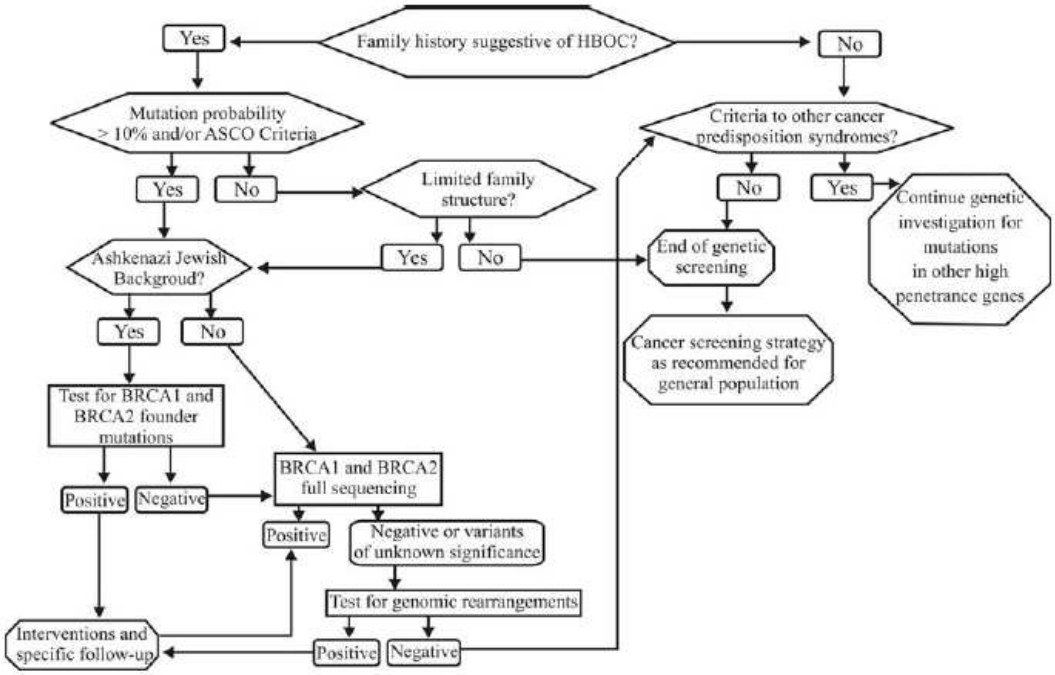

Figure 2 - Suggested approach for molecular investigation of hereditary breast and ovarian cancer (HBOC) families. The mutation probabilities are estimated by using standard protocols and/or risk estimation tools such as BRCAPro, BOADICEA and the Myriad mutation prevalence tables. ASCO: American Society of Clinical Oncology.

ward screening methods has greatly simplified this process, but often more than one method must be used to fully characterize a deletion or duplication in a given patient. Several studies in different populations have proven the usefulness of screening for *BRCA1* rearrangements, however the prevalence of such mutations in a given population should be known before definitive recommendations are made regarding the routine testing for rearrangements. In populations where there are highly prevalent founder rearrangements, preliminary screening for pathogenic *BRCA* gene mutations may be a cost-effective initial strategy.

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Internet Resources

Multiplex ligation-dependent probe amplification: http://www.mrc-holland.com/pages/p002pag.html (July 19, 2008).

6. MANUSCRITO 2: International distribution and age estimation of the Portuguese *BRCA2* c.156_157insAlu founder mutation.

International distribution and age estimation of the Portuguese *BRCA2* c.156_157insAlu founder mutation

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Abstract The c.156_157insAlu *BRCA2* mutation has so far only been reported in hereditary breast/ovarian cancer (HBOC) families of Portuguese origin. Since this mutation is not detectable using the commonly used screening methodologies and must be specifically sought, we screened for this rearrangement in a total of 5,443 suspected HBOC families from several countries. Whereas the c.156_157insAlu *BRCA2* mutation was detected in 11 of 149 suspected HBOC families from Portugal, representing 37.9% of all deleterious mutations, in other countries it was detected only in one proband living in France and in four individuals requesting predictive testing living

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in France and in the USA, all being Portuguese immigrants. After performing an extensive haplotype study in carrier families, we estimate that this founder mutation occurred 558 ± 215 years ago. We further demonstrate significant quantitative differences regarding the production of the *BRCA2* full length RNA and the transcript lacking exon 3 in c.156_157insAlu *BRCA2* mutation carriers and in controls. The cumulative incidence of breast cancer in carriers did not differ from that of other *BRCA2* and *BRCA1* pathogenic mutations. We recommend that all suspected HBOC families from Portugal or with Portuguese ancestry are specifically tested for this rearrangement.

Keywords c.156_157insAlu *BRCA2* mutation · Founder mutation · Age estimation · Hereditary breast/ovarian cancer

Introduction

The pattern of *BRCA1* and *BRCA2* mutations in hereditary breast/ovarian cancer (HBOC) families varies widely among different populations. Many present a wide spectrum of different mutations throughout these genes, while some ethnic groups show a high frequency of particular mutations due to founder effects [1, 2]. Identification of founder mutations makes it possible to use more specific approaches to molecular testing [3], allowing the analysis of more patients with less stringent selection criteria in a given population. Furthermore, a frequent founder mutation in a population allows a more accurate estimation of mutation-specific cumulative cancer incidence, facilitating also identification of genetic and environmental risk modifiers.

The c.156_157insAlu *BRCA2* mutation was first described by Teugels et al. [4] in a Portuguese patient residing in Belgium. These authors demonstrated that this exon 3 Alu insertion causes an in-frame deletion of that exon at the mRNA level, and thereby deletes a transcriptional activation domain [4]. Machado et al. [5] later described a regional founder effect for this rearrangement in HBOC families mostly originated from central/southern Portugal. We recently evaluated the contribution of the c.156_157insAlu *BRCA2* mutation to inherited predisposition to breast/ovarian cancer in families originated mostly from northern/central Portugal [6] and found that this

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rearrangement is responsible for more than half of all deleterious *BRCA2* mutations and about one-fourth of all deleterious mutations in HBOC families. Additionally, in light of some doubts raised about the pathogenic effect of *BRCA2* exon 3 skipping [7], we demonstrated that the *BRCA2* full length transcript is produced exclusively from the wild type allele in patients carrying the c.156_157insAlu *BRCA2* rearrangement and that the mutant allele co-segregates with the disease in HBOC families and is absent in healthy blood donors, although minimal exon 3 skipping in *BRCA2* mRNA can be found in negative controls [6, 8].

Although all reported c.156_157insAlu *BRCA2* mutations have so far been identified in Portuguese HBOC families [4–6], this mutation is not detected using the common screening methodologies and must be specifically sought [4, 8], so one cannot currently rule out its presence in other populations. To gain insight into the ancestral origin and population spread of the c.156_157insAlu *BRCA2* mutation, we screened for this rearrangement in 5,443 suspected HBOC patients from several countries and performed an extensive haplotype study using closely linked microsatellite markers and single nucleotide polymorphisms (SNPs) in carrier families. In addition to estimating the age of the c.156_157insAlu *BRCA2* mutation, we used real-time RT-PCR to quantify the production of the transcript lacking exon 3 in carriers and non-carriers.

Materials and methods

Families

This study comprised a total of 5,443 suspected HBOC families from 13 countries in Europe, North and South America and Asia. From Portugal, 149 new suspected HBOC families were selected for *BRCA1* and *BRCA2* mutation screening using previously described criteria [6, 9] after written informed consent. Molecular testing at the Department of Genetics of the Portuguese Oncology Institute, Porto, Portugal (IPO-Porto) started by looking for the c.156_157insAlu *BRCA2* mutation, followed by full *BRCA1* and *BRCA2* mutation screening with the previously reported methodology [6, 8, 9]. Additionally, screening for the c.156_157insAlu *BRCA2* mutation was performed in 5,294 suspected HBOC families living in countries other than Portugal in whom no deleterious *BRCA1*/*BRCA2* mutations had previously been found, with the following

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distribution: 1,209 from Spain (356 from L'Hospitalet de Llobregat, 341 from Madrid, 151 from Valladolid, 132 from Zaragoza, 123 from Santiago de Compostela, and 106 from Barcelona), 1,087 from France (650 from Clermont-Ferrand, 428 from Saint-Cloud, and nine from Villejuif, all the latter with Portuguese ancestry), 820 from Holland (Groningen), 758 from Denmark (Funen and Jutland), 400 from Greece (Athens), 219 from Switzerland (Geneva), 200 from Belgium (Brussels), 185 from Israel (Tel Aviv), 144 from Brazil (98 from Porto Alegre and 46 from S. Paulo), 103 from Canada (Montreal), 91 from India (Chennai), 75 from Italy (Rome), and three from USA (Seattle, all with Portuguese ancestry). Besides the suspected HBOC families, two consecutive series of breast cancer patients from Rio de Janeiro, Brazil (390), and Azorean Island of São Miguel, Portugal (86), were also screened for the c.156_157insAlu *BRCA2* mutation. Additionally, predictive testing was performed in four individuals from two additional families (two relatives from each family living in Rhode Island, USA, and in Villejuif, France, respectively) with the c.156_157insAlu *BRCA2* mutation identified elsewhere. IRB approval was obtained at each participating institution.

For the purpose of haplotype studies and age estimation of the c.156_157insAlu *BRCA2* mutation, the 14 HBOC families we previously reported [6] and the family (four c.156_157insAlu carriers) initially identified by Teugels et al. [4] were also included. The geographic origin of the c.156_157insAlu *BRCA2* positive families was inferred from the birthplace of the oldest carrier or of the oldest family member most likely to be a carrier.

Screening for the c.156_157insAlu *BRCA2* mutation

The screening for the c.156_157insAlu *BRCA2* mutation in the suspected HBOC families from Portugal, and of samples originating from the Athens, Barcelona, Madrid and Zaragoza labs, as well as the predictive testing of four individuals from two additional families living in Rhode Island and Villejuif, respectively, was performed at the Department of Genetics of IPO-Porto. The remaining cases were analyzed at the respective labs (except the cases from Rio de Janeiro, which were analyzed in Toronto) using the same protocol and a positive control provided by the Portuguese lab.

Screening for the c.156_157insAlu *BRCA2* mutation was performed using two independent PCRs [6, 8], one for exon 3 amplification and another specific for the Alu rearrangement. Using this strategy, we expect two amplicons in positive cases in the first PCR (one amplicon if negative) and one amplicon in the second PCR (none if negative). The second PCR helps to control the first PCR for potential problems with preferential amplification of the shorter fragment (wild type), whereas the first PCR controls for potential absence of amplification in the second PCR. This strategy of two independent PCRs, followed by sequencing of the genomic fragments in positive cases, allows the unambiguous detection of the c.156_157insAlu *BRCA2* mutation [6, 8]. Positive and negative controls were used in all experiments and all positive cases were confirmed in a second independent sample.

Real-time RT-PCR analysis

Primers and probes for the transcripts *BRCA2* wild type (*BRCA2*-wt) and *BRCA2* lacking exon 3 (*BRCA2*-Δex3) were designed with Primer Express 2.0 (Applied Biosystems, Foster City, USA) (Supplementary file). To determine the relative expression levels of the target transcripts in each sample, the comparative C_T method was performed as described by Schmittgen and Livak [10]. The relative expression of the transcripts in two different groups (that included 10 carriers and eight controls) was calculated using the $2^{-\Delta C_T}$ method. The ratio $2^{-\Delta C_T}$*BRCA2*-Δex3/$2^{-\Delta C_T}$*BRCA2*-*wt* was calculated for each sample. The Mann–Whitney *U* Test was used to compare the relative expression of those transcripts between the two groups. Statistical analysis was performed with SPSS version 11 and statistical significance was considered whenever $P < 0.05$.

Mutation-specific cumulative incidence of breast cancer

The cumulative incidence of breast cancer in women with the c.156_157insAlu *BRCA2* mutation was derived using the method of Kaplan and Meier, with unaffected individuals censored at the age of last follow-up or death without breast cancer. Only individuals shown to be carriers or obligate carriers were used for this calculation.

Microsatellite and SNP typing

Haplotype analysis was carried out in families in which the c.156_157insAlu *BRCA2* mutation was detected in at least one family member in addition to the proband. A total of 15 probands and 62 family members, including the three informative families previously reported [6] and the one described by Teugels et al. [4], were genotyped for polymorphic microsatellite markers flanking *BRCA2* as described [6]. The physical distances of the genetic markers were derived from the National Center for Biotechnology Information (NCBI) Map Viewer (genome build 36.3) (http://www.ncbi.nlm.nih.gov/projects/mapview/). All nine markers were assayed by PCR using fluorescently 5′-labeled primers. PCR products were run on an ABI PRISM 310

Genetic Analyser (Applied Biosystems) together with the fluorescence labeled DNA fragment size standard TAMRA.

Single-nucleotide polymorphism (SNP) markers were used to obtain a haplotype spanning ~1.1 Mb encompassing the region between the D13S260 and D13S1695 microsatellite markers, where the first recombinant and/or mutational events were observed. In order to capture most of the genetic variation in this region and to avoid redundant SNP markers (i.e., markers in strong linkage disequilibrium), we performed Tag-SNP, namely Tagger Multimarker, using International HapMap Project CEPH (Utah residents with ancestry from northern and western Europe) population data (www.hapmap.org). We developed SNaPshot assays for 19 SNP markers by multiplexed nucleotide primer extension reaction using dye label terminators (Applied Biosystems). The primers for multiplex amplification and single base extension (Supplementary file) were designed using the online Primer-BLAST tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). AutoDimer (www.cstl.nist.gov/strbase/NIJ/AutoDimer.htm) was used to test for potential hairpin structures and primer dimers. The 19 SNPs were PCR amplified in four multiplex reactions with amplicon length between 100 and 450 bp. The multiplex SNaPshot reaction and capillary electrophoresis was done following the manufacturer's protocol (Applied Biosystems).

Haplotype construction and estimation of mutation age

Haplotype construction was performed manually based on the genotypes obtained of index cases and family members. We estimated the age of the c.156_157insAlu *BRCA2* mutation from the variation accumulated in their ancestral haplotypes, as described by Martins et al.[11] This method takes into account both recombination (c) and mutation (μ) rates in the generation of variation. The probability of change per generation (ε) is given by $\varepsilon = 1-[(1-c)(1-\mu)]$, and the average of mutation and recombination events (λ) equals εt, where t is the number of generations. The recombination rate (c) was estimated from the physical distance between the two most distant markers (D13S1700 and D13S267) using a conversion factor calculated in Rutgers Map Interpolator (http://compgen.rutgers.edu/old/map-interpolator/). The estimate of average mutation rate used was 7.8×10^{-4} [12] for dinucleotides and two times lower for tetranucleotides.

Results

Detection of the c.156_157insAlu *BRCA2* mutation

Of the 149 Portuguese probands studied for germline mutations in the *BRCA1* and *BRCA2* genes at IPO-Porto, 11 patients presented the c.156_157insAlu *BRCA2* mutation (Fig. 1) and 18 patients presented other deleterious mutations in either *BRCA1* (10 patients) or *BRCA2* (8 patients) genes (data not shown). Together with the 14 probands we previously reported with this mutation [6], a total number of 25 HBOC families with the c.156_157insAlu *BRCA2* rearrangement had been identified at IPO-Porto at the time of writing. Altogether, 68 individuals from these 25 HBOC families have so far been tested for the c.156_157insAlu *BRCA2* mutation and 39 of them were shown to be carriers of the mutant allele. The geographic origins of all the c.156_157insAlu *BRCA2* positive families are shown in Supplementary Fig. 1. Although most of the families originated from northern/central Portugal, most likely reflecting our target population for genetic testing, we also detected the c.156_157insAlu mutation in families from southern Portugal and Madeira Island.

Of the 5,294 suspected HBOC families with no known deleterious mutation originating from other countries, only one proband tested in Clermont-Ferrand was shown to carry the c.156_157insAlu *BRCA2* mutation. Interestingly, this patient belongs to a family of Portuguese origin living in France. Additionally, the two relatives living in Rhode Island (family with origin in Mangualde, central Portugal) and the two relatives living in Villejuif (family with origin in Porto, Portugal) for whom we performed predictive testing, were carriers of the c.156_157insAlu *BRCA2* mutation that had previously been identified elsewhere in Portuguese family members. Finally, the patient originally reported by Teugels et al. [4] belongs to a Portuguese family originally from the region of Guarda (central Portugal).

Quantitative transcript analysis

Real-time RT-PCR showed quantitative differences between the full length and the *BRCA2*-Δex3 transcripts in c.156_157insAlu *BRCA2* mutation carriers and controls. The relative expression of the *BRCA2*-Δex3 transcript was sixfold higher in carriers compared with controls, whereas a threefold decrease was observed for the *BRCA2*-wt transcript in patients compared with controls (Fig. 2). The difference observed between patients and controls was statistically significant ($P = 0.00032$).

Mutation-specific cumulative incidence

Using the method of Kaplan and Meier, the cumulative incidence of breast cancer in women carrying the c.156_157insAlu *BRCA2* mutation was 90% until the age of 60 years (Fig. 3).

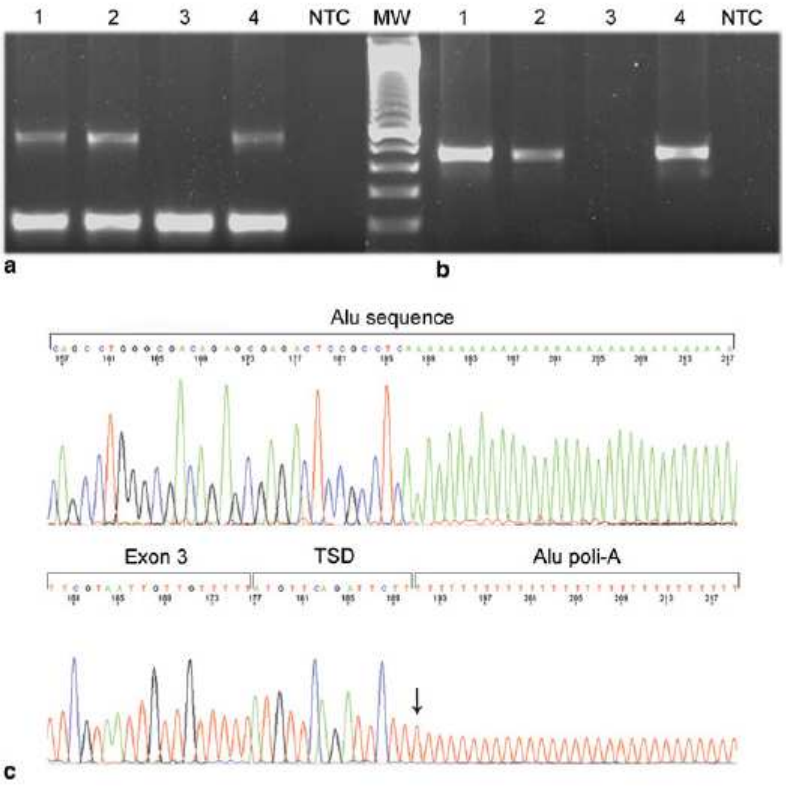

Fig. 1 Molecular diagnosis of the *BRCA2* c.156_157insAlu mutation using two independent PCR analyses, showing positive cases in *lanes 1* and *2* and a negative case in *lane 3*. *Lane 4* corresponds to a positive control and NTC is a non template control. MW refers to 100 bp DNA standard. (**a**) PCR specific for *BRCA2* exon 3, showing an additional band resulting from the insertion of a DNA fragment of about 350 bp long within exon 3 of *BRCA2* in positive cases. (**b**) PCR specific for the c.156_157insAlu *BRCA2* mutation, showing an amplicon in positive cases. (**c**) Sequence electrophorograms of the amplified genomic fragment of a mutation positive case (forward, top; reverse, bottom), confirming the Alu insertion (*arrow*) in *BRCA2* gene exon 3. The Alu insertion is flanked by a short sequence duplication (TSD) as previously described by Teugels et al. [4]

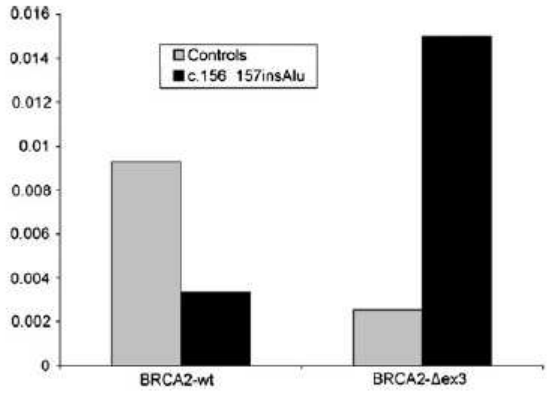

Fig. 2 Real-time RT–PCR quantification of the altered transcript ratios in *BRCA2* c.156_157insAlu carriers as compared with controls. The relative expression of the *BRCA2*-Δex3 transcript was sixfold higher in carriers compared with controls, whereas a threefold decrease was observed for the *BRCA2*-wt transcript in patients compared with controls

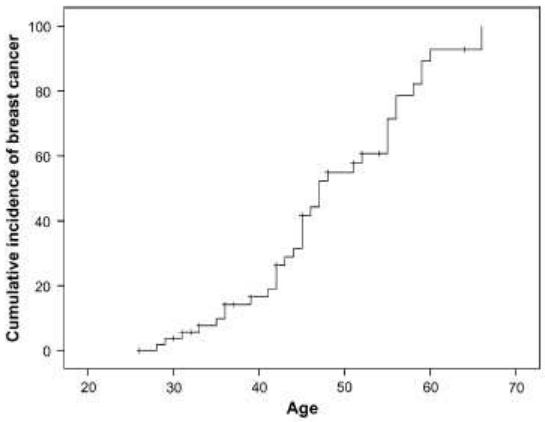

Fig. 3 Cumulative incidence of breast cancer among c.156_157insAlu *BRCA2* germline mutation carriers, reaching 90% at about 60 years of age

Ancestral STR-based haplotypes and age estimate

Nine different haplotypes were phased for 11 out of the 15 families, three of them reported earlier [6]. The results of the haplotype analyses for the 11 informative families are shown in Table 1 and the most parsimonious relationships among flanking haplotypes are presented as a phylogenetic network in Fig. 4. The probability of mutation versus recombination was evaluated, considering the minimum number of stepwise mutations. In the 11 informative

Table 1 Age estimation of the ancestral c.156_157insAlu *BRCA2* mutation

Haplotype[a]	Families, No.	Mutation steps/recombination events, No.	Age ± δ, y[b]
H1: **317-160-156-299-230-242-228-144-158**	2	0	558 ± 215
H2: **317-160-156**-295-**230-242-228-144-158**	1	1	
H3: 313-**160-156-299-230-242-228-144-158**	1	1	
H4: 269-**160-156-299-230-242-228-144-158**	1	1	
H5: **317-160-156-299-230**-234-234-148-144	2	1	
H6: 321-**160-156-299-230**-234-234-148-144	1	2	
H7: **317-160-156**-307-226-252-234-148-144	1	1	
H8: **317-160-156-299-230**-252-234-144-156	1	1	
H9: 309-162-160-**299-230-242-228-144-158**	1	1	
Total	11	9	

[a] The nine microsatellite markers used were: D13S1700, D13S260, D13S1698, D13S1701, D13S171, D13S1695, D13S1694, D13S310, and D13S267 (from left to right). Ancestral haplotype in which Alu insertion probably occurred is indicated in bold

[b] The recombination rate (c) was based on the physical distance between the two most distant markers (1930.8 kb; $c = 0.030597$ cM) using a conversion factor calculated in Rutgers Map Interpolator. The estimated probability of mutation per generation and per haplotype was 0.00624 (as seven dinucleotide and two tetranucleotide short tandem repeats were studied)

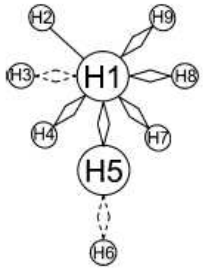

Fig. 4 Phylogenetic network showing the most parsimonious relationships among flanking short tandem repeat-based haplotypes in families carrying the c.156_157insAlu *BRCA2* mutation. *Circle* and *line* sizes are proportional to the number of families and stepwise mutations, respectively. *Diamonds* indicate recombination events. When it was not possible to determine if the most parsimonious relationship was due to a stepwise mutation or a recombination event we represented it by *dashed diamonds*

families, SNP haplotypes were constructed in order to establish if a specific microsatellite was different from the consensus because of a recombination event rather than a mutation (Supplementary Fig. 2).

Based on the mutation and recombination events observed in microsatellite haplotypes and assuming a generation time of 25 years, the age estimate for the c.156_157insAlu *BRCA2* mutation is 558 ± 215 years (Table 1).

Discussion

The c.156_157insAlu *BRCA2* mutation has so far only been reported in HBOC families of Portuguese origin. Here we show that this rearrangement accounts for 57.8% of the *BRCA2* mutations and 37.9% of all deleterious mutations in HBOC families originating mostly from northern/central Portugal. This study confirms our and other earlier findings indicating that this is by far the most common BRCA mutation in Portuguese families with hereditary predisposition to breast/ovarian cancer, being detected in about 8% of all probands tested and presenting a nation-wide distribution [5, 6]. This high frequency makes it cost-effective to test specifically for this rearrangement prior to screening the entire coding regions of *BRCA1* and *BRCA2* in suspected HBOC families from Portugal or with Portuguese ancestry. Furthermore, complementing earlier data showing that the c.156_157insAlu *BRCA2* mutation leads to skipping of exon 3 [4] and that minimal exon 3 skipping in *BRCA2* mRNA can be found in negative controls [6, 8], we here demonstrate by real-time RT-PCR that carriers present significantly more *BRCA2*-Δex3 transcripts and much less full length transcripts than controls. We further show that the cumulative incidence of breast cancer in c.156_157insAlu *BRCA2* mutation carriers does not differ from that of other *BRCA2* and *BRCA1* pathogenic mutations in our population (data not shown) or elsewhere [13], further strengthening its role as the major contributor to hereditary predisposition to breast cancer in Portugal. Although the function, if any, of the *BRCA2* exon 3 skipping seen in controls is unknown, the observed penetrance in c.156_157insAlu carriers would not be expected if this transcript was fully functional. We therefore conclude that this *BRCA2* rearrangement causes hereditary breast/ovarian cancer because the mutated allele is only able to give

rise to *BRCA2*-Δex3 transcripts and not to *BRCA2*-wt transcripts.

Since the c.156_157insAlu *BRCA2* mutation had only been reported in HBOC families of Portuguese origin [4–6] and is not detectable with commonly used screening methodologies, one can not exclude that it is present in other populations until it is specifically sought. To further evaluate whether or not it constitutes a population-specific founder mutation, we screened for the c.156_157insAlu *BRCA2* rearrangement outside Portugal in more 5,294 suspected HBOC families with no known deleterious *BRCA1*/*BRCA2* mutations coming from several countries mainly from Europe, but also from Asia and North and South America. In addition to the family identified in Belgium by Teugels et al. [4], we now detected this mutation in one proband living in France and in four individuals requesting predictive testing living in France and in the USA, all having in common the fact that they are relatively recent immigrants of Portuguese origin in those countries. Interestingly, c.156_157insAlu *BRCA2* mutation was not detected in 1,209 suspected HBOC families from Spain, including those from Galicia, the Spanish region with which Portugal shares more linguistic and cultural links, as also demonstrated by our recent finding of a common ancestry for the Portuguese HBOC families presenting the R71G *BRCA1* founder mutation of Galician origin [14].

Our findings indicate that, within the relatively large sample population studied, the c.156_157insAlu *BRCA2* mutation is unique to HBOC families of Portuguese ancestry, a fact that is hardly compatible with the age of about 2500 years previously estimated by Machado et al. [5]. Although geographic distribution of mutations is only an indirect measure of mutation age, more widespread mutations tend to be older than mutations showing a regional distribution, with the development of urbanization and industrialization in the past 700 years leading to rapid populations growth and therefore to the recent appearance of vast numbers of new alleles, some of which cause hereditary breast/ovarian cancer, each being specific to one population or even to one family [15]. In order to get a more accurate mutation age estimate of the c.156_157insAlu *BRCA2* rearrangement, we performed an extensive haplotype analysis having in mind that the size of an ancestral haplotype around a mutation is inversely correlated with the number of generations separating the common ancestor from the families carrying that rearrangement. After performing the haplotype reconstruction in the 11 informative families and assuming a generation time of 25 years, we estimate the age of the c.156_157insAlu *BRCA2* mutation to be 558 ± 215 years, that is, most likely well after Portugal became politically independent (in 1143). Our estimate is consistent with the widespread distribution of the mutation in Portugal [5, 6], the country demographic history (the North has been and still is consistently the source of migrants to the South), its occasional finding in countries with strong Portuguese immigration, and with its absence in the other populations studied (e.g., absence of the mutation in Spain, namely in Galicia). Nevertheless, statistical methods for estimating mutation ages are relatively crude [16], are dependent on sample representativeness, and estimate only the age of the common ancestor to the informative families that have been identified. The older age estimate advanced by Machado et al. [5] was based upon a different sample of Portuguese patients (mostly from Center and South) and using a different age estimate method. However these authors recognize that the age of the mutation may be «overestimated, either because of the fact that mutation rates of the microsatellite markers were not taken into account or because recombination events in two families were considered». On the other hand, although the mutation has so far only been detected in Portugal and in a few families with Portuguese ancestry living in Belgium, France or the USA, we can not conclusively exclude its presence in other countries that have strong historical links with Portugal, such as those having Portuguese as official language (Brazil, Angola, Mozambique, Cape Verde, Guinea-Bissau, São Tomé and Príncipe, East Timor, and Macau) or other countries with a large community of Portuguese immigrants. In fact, one of our probands with the c.156_157insAlu *BRCA2* mutation illustrates this possibility: although she is now living in Portugal, her ancestors originating from North Portugal had moved several generations ago to Brazil and later to Angola, where reportedly various affected relatives lived.

In conclusion, we showed that the c.156_157insAlu *BRCA2* rearrangement is a Portuguese founder mutation originated about 558 ± 215 years ago, accounting for the majority of the *BRCA2* mutations and for about one-third of all deleterious germline mutations in Portuguese HBOC families. We therefore recommend that all suspected HBOC families from Portugal or with Portuguese ancestry are specifically tested for this rearrangement, ideally prior to screening the entire coding regions of *BRCA1* and *BRCA2*. We further showed that the cumulative incidence of breast cancer in c.156_157insAlu *BRCA2* mutation carriers does not differ from that of other *BRCA2* and *BRCA1* pathogenic mutations and that this *BRCA2* rearrangement causes hereditary breast/ovarian cancer because the mutated allele is only able to give rise to *BRCA2*-Δex3 transcripts and not to *BRCA2*-wt transcripts.

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7. MANUSCRITO 3: Li-Fraumeni-like syndrome associated with a large *BRCA1* intragenic deletion

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Li-Fraumeni-like syndrome associated with a large BRCA1 intragenic deletion

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Abstract

Background - Li-Fraumeni (LFS) and Li-Fraumeni-like (LFL) syndromes are associated to germline TP53 mutations, and are characterized by the development of central nervous system tumors, sarcomas, adrenocortical carcinomas, and other early-onset tumors. Due to the high frequency of breast cancer in LFS/LFL families, these syndromes clinically overlap with hereditary breast cancer (HBC). Germline point mutations in BRCA1, BRCA2, and TP53 genes are associated with high risk of breast cancer. Large rearrangements involving these genes are also implicated in the HBC phenotype.

Methods - We have screened DNA copy number changes by MLPA on BRCA1, BRCA2, and TP53 genes in 23 breast cancer patients with a clinical diagnosis consistent with LFS/LFL; most of these families also met the clinical criteria for other HBC syndromes.

Results - We found no DNA copy number alterations in the BRCA2 and TP53 genes, but we detected in one patient a 36.4 Kb BRCA1 microdeletion, confirmed and further mapped by array-CGH, encompassing exons 9-19. Breakpoints sequencing analysis suggests that this rearrangement was mediated by flanking Alu sequences.

Conclusion - This is the first description of a germline intragenic BRCA1 deletion in a breast cancer patient with a family history consistent with both LFL and HBC syndromes. Our results show that large rearrangements in these known cancer predisposition genes occur, but are not a frequent cause of cancer susceptibility.

Keywords: breast cancer, copy number variation, MLPA, BRCA1 microdeletion, Li-Fraumeni syndrome

Background

Germline mutations of the tumor suppressor gene TP53 account for more than half of the families with classic Li-Fraumeni syndrome (LFS) [1], which is an inherited condition characterized by the development of sarcomas and other early-onset tumors, including breast cancer [2, 3]. Families presenting incomplete features of LFS are referred as having Li-Fraumeni-like syndrome (LFL). Depending on the criteria adopted to classify the cancer phenotype in a given family, up to 22% of LFL pedigrees have detectable TP53 mutations [4-6]. Several cancer predisposition syndromes that involve breast cancer have been described to date, and include, in addition to LFS/LFL, the hereditary breast and ovarian cancer (HBOC), hereditary diffuse gastric cancer, and the Cowden and Peutz-Jeghers syndromes [7]. Due to the high frequency of breast and other cancers in LFS/LFL individuals, there may be an overlap of phenotypes, and often some families fulfill genetic testing criteria for more than one hereditary breast cancer syndrome [1, 8, 9]. Several studies have investigated the frequency of BRCA1/BRCA2 and TP53 germline mutations in families with multiple early-onset breast cancers [6, 8, 10,11]. Approximately 5-10% of breast cancer is estimated to result from dominant mutations in known single genes [12-14], particularly in the BRCA1 or BRCA2 genes. Germline TP53 mutations have been considered to be responsible for only a small fraction of the hereditary breast cancer cases overall [15], and have mostly been described in families with the other core-cancers of LFS/LFL [1, 8,9].Germline mutations of the BRCA2 gene have been described in families presenting both breast cancer and sarcomas, suggesting that BRCA2 mutations account for a proportion of LFS/LFL families negative for TP53 mutations [16,17]. As far as we are aware, germline BRCA1 mutations have not been detected in LFS/LFL kindreds, not even among families presenting a complex cancer history consistent both with LFL and other syndromes that constitute the HBC phenotype [6, 8, 11, 18]. All known breast cancer susceptibility genes present germline point mutations in only approximately 20-25% of the cases fulfilling the criteria for genetic testing [12]. Gene rearrangements can contribute to disease through different mechanisms, resulting in either imbalance of gene dosage or gene disruption, and they are not usually detected by routine molecular diagnostic methods such as gene sequencing. In particular, large rearrangements, most often deletions, have been reported as a cause of cancer susceptibility, occurring in at least 30% of highly penetrant Mendelian cancer-predisposing genes [19]. BRCA1 germline rearrangements have been implicated in up to 30% of HBC families in certain populations [19-23]. The aim of the present study was to determine the frequency of germline copy number changes of TP53, BRCA1, and BRCA2 genes in breast cancer patients with clinical diagnosis

of Li- Fraumeni or Li-Fraumeni-like syndrome, and without detectable germline TP53 point mutations.

Material and Methods

Patients

The research protocol was approved by the institutional ethics committees of the participating Institutions (Protocol numbers 1175/08 and GPPG-HCPA 04-081), and recruitment of patients was done after signature of informed consent. DNA samples from 23 patients were obtained from peripheral blood; sample quality was assessed using Nanodrop and molecular weight was checked by electrophoresis in 0.8% agarose gels. TP53 mutation testing was previously performed by direct sequencing of exons 2-11, using the protocols published in http://www-p53.iarc.fr/p53sequencing.html[24].

Family history was recorded in detailed pedigrees with information traced as far backwards and laterally as possible, extending to paternal lines and including a minimum of three generations. Confirmation of the family history of cancer was attempted in all cases and pathology reports, medical records and/or death certificates were obtained whenever possible. We selected 23 breast cancer patients with an indication for TP53 mutation testing due to a Li-Fraumeni or Li-Fraumeni-like phenotype according to the classical criteria [32] or at least one of the LFL definitions: Chompret, Birch or Eeles [4, 33-35]. In all families, TP53 mutation testing was negative [36]. Additionally, some of these families also fulfilled mutation testing criteria for other hereditary breast cancer syndromes, as described in the NCCN Practice Guidelines in Oncology – v.1.2010[37]. Clinical features of the 23 probands are summarized in Table 1.

Multiplex ligation probe amplification (MLPA)

Deletions and duplications affecting all coding exons of the TP53 gene (12 probes) were investigated by MLPA (MRC-Holland, Amsterdam, The Netherlands, kit P056) [38]. MLPA experiments were performed in duplicates for each patient sample, with simultaneous analysis of DNA samples from two healthy individuals from the general population (negative controls), and two patients carrying previously characterized germlineTP53 rearrangements (positive controls: a Li-Fraumeni patient with an intragenic TP53 deletion [39]; and a patient harboring a large 17p13 duplication from our in-house database). Deletions and duplications affecting BRCA1 and BRCA2 exons were also investigated by MLPA (MRC-Holland, Amsterdam, The

Netherlands, kits P087 and P045, respectively; kit P002 was also used for confirmatory analysis of one detected BRCA1 microdeletion); duplicated experiments were performed simultaneously in samples from patients, two healthy individuals, and samples previously identified as carrying large duplications encompassing the BRCA1and BRCA2 genes (positive controls; patients from our in-house database). The PCR-amplified fragments were separated by capillary electrophoresis on an ABI 3130 XL genetic analyzer (Applied Biosystems, Foster City, California), and analyzed using the Coffalyser software (MRC Holland). We performed direct normalization with control probes as normalization factor, using the median of all imported samples, and two standard deviations. Values >1.3 were considered as possible duplications, and deletions were considered for probes exhibiting values < 0.7. Using this analysis, alterations present in all positive controls were detected.

Comparative genomic hybridization on microarrays (array-CGH)

Array-CGH analysis was performed as previously described [40] to confirm a intragenic BRCA1 deletion detected by MLPA in one patient (Y54). We used a whole-genome 180K platform (Agilent Technologies), according to the manufacturer's instructions; a gain or loss in copy number was considered when the log2 ratio of the Cy3/Cy5 intensities of a given genomic segment was > 0.6 or < -0.8, respectively. As reference DNA, we used commercially available human Promega female DNA (Promega, Madison, WI, USA).

Breakpoint Sequencing Analysis

To assess the microdeletion breakpoints, specific primers (forward: 5'-ACTCTGAGGACAAAGCAGCGGA -3'; reverse: 5'- GTGCCACCAAGCCCGGCTAA -3') were designed in order to amplify the breakpoint region of the BRCA1 rearrangement (microdeletion involving the same exons described by [20]. A 450 bp fragment was detected only in the sample with the microdeletion, and absent in the normal controls. The 450 bp fragment was purified from the gel using the Gel Band Purification Kit (Illustra, GE Healthcare UK limited, Buckinghamshire, United Kingdom) and sequenced (forward and reverse) using the Big Dye V3.1 Terminator Kit (Applied Biosystems, Forster City, CA, USA) on an automated sequencer ABI Prism 310 Genetic Analyser (Applied BioSystems,) according to the manufacturer's instructions. We performed an in silico analysis of the genomic sequences surrounding the breakpoints using the RepeatMasker program (http://www.repeatmasker.org/) that screens DNA sequences for interspersed repeats and low complexity DNA sequences.

Results

All studied patients were females affected by breast cancer, two of them with bilateral disease, and 11 (45.8%) with more than one primary tumor. The average age at breast cancer diagnosis was 41 years (SD: 11.5; range: 26 - 61 years). Nineteen of the 23 families met genetic testing criteria for both LFL and another hereditary breast cancer syndrome (Table 1); two families met criteria for both classic LFS and another hereditary breast cancer syndrome, and two fulfill only the criteria for LFL. In the MLPA analysis none of the patients showed TP53, or BRCA2 deletions or duplications. We identified a single patient carrying a heterozygous intragenic BRCA1 microdeletion (Y54). Analysis using two different sets of MLPA probes (kits P087 and P002) and array-CGH allowed confirming a deletion that spanned from exon 9 to 19 (Figure 1 depicts the chromosome 17q21.31 array- CGH profile of the patient, indicating the position of the BRCA1 microdeletion). We tested two non-affected relatives of patient Y54 (III.13 and III.16) and found that one of them carries the BRCA1 deletion (III.16). Unfortunately, affected relatives of the patient Y54 could not be investigated for the presence of the BRCA1 deletion either because they were deceased or were not available. The DNA fragment containing the rearrangement breakpoints was sequenced and the results showed that the deletion starts at intron 8 and ends at intron 19 of the BRCA1 gene, resulting in a deletion-block identified as: g.29197_65577del36381 (Figure 2). Detailed in silico assessment of the genomic sequences surrounding the breakpoints showed that consensus Alu
sequences flanked them. Clinically, this family fulfilled genetic testing criteria for both hereditary breast and ovarian cancer (HBOC) and LFL (Eeles 1 criteria) syndromes; the cancer family history was significant for the presence of two individuals with multiple primary tumors, including the proband (Figure 3).

Discussion

In families with a breast cancer history that suggests the involvement of high risk genes such as TP53, BRCA1 and BRCA2, a more extensive analysis of these genes should be considered. In this study we have screened three major breast cancer predisposition genes for copy number changes in a group of 23 breast cancer patients with the clinical diagnosis of LFS/LFL who had no germline TP53 point mutations. We did not identify large rearrangements encompassing TP53, which is in line with previous reports of low prevalence of such alterations, encountered in less than 5% of LFS/LFL families [24, 25].
Similarly, large rearrangements in other breast cancer predisposition genes seem to be infrequent. A few BRCA2 deletions have been previously reported in families with male breast

cancer [26], and contribute to inactivate this gene in breast cancer families [21, 27]. Rearrangements affecting the BRCA2 gene have also been reported in breast/sarcoma families, causing a Li–Fraumeni type of cancer pattern [16]. Although none of the families included in this study had male breast cancer cases, nine of them had a breast cancer/sarcoma phenotype; however, no BRCA2 rearrangements were identified, which may be related to the relatively small sample size. BRCA1 rearrangements, on the other hand, are more prevalent mostly due to the high density of Alu elements throughout the BRCA1 locus [28]. A large study by Walsh et al (2006)[11] suggested that the mutation spectra of BRCA1/BRCA2 includes several genomic rearrangements, and those alterations seem to be particularly frequent in certain populations (due to founder effect), and in families presenting individuals with multiple primary tumors [20, 21, 29, 30]. Indeed, the "multiple primary tumors" phenotype was observed in the BRCA1 rearrangement-positive family identified in our series. Interestingly, the BRCA1 microdeletion identified here appears to be the same as the one identified in a breast cancer Italian patient [20]. Our patient is originally from southern Brazil, and since Italians have strongly contributed to the ethnic make-up of southern Brazilian population [31] it is possible that the Brazilian and the Italian patients have a common ancestry. Considering that we could not establish the parental origin of the rearrangement, this large genomic deletion may represent a breast cancer susceptibility allele rather than a more general cancer predisposition factor. This study contributes to the understanding of the etiology of cancer susceptibility in Li-Fraumeni (LFS) and Li-Fraumeni-like (LFL) families, and their possible relation to large genomic rearrangements in high risk breast cancer susceptibility genes.

Conclusion

In patients with a cancer family history consistent with genetic testing criteria for multiple breast cancer syndromes, a comprehensive investigation, including full gene sequencing and rearrangement screening of multiple loci may be necessary to determine the precise molecular mechanisms underlying the disease. However, as illustrated with this study, in many families with cancer histories clearly indicative of hereditary cancer predisposition, the disease-causing molecular mechanisms remain elusive. Thus, despite the availability of extensive genotyping and sequencing approaches, determination of the precise pathogenic mechanisms of hereditary cancer in many cases is still a significant challenge.

Material and Methods

Patients

The research protocol was approved by the institutional ethics committees of the participating Institutions (Protocol numbers 1175/08 and GPPG-HCPA 04- 081), and recruitment of patients was done after signature of informed consent. DNA samples from 23 patients were obtained from peripheral blood; sample quality was assessed using Nanodrop and molecular weight was checked by electrophoresis in 0.8% agarose gels. TP53 mutation testing was previously performed by direct sequencing of exons 2-11, using the protocols published in http://www-p53.iarc.fr/p53sequencing.html[24].

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List of abbreviations

LFS - Li-Fraumeni syndrome
LFL - Li-Fraumeni like syndromes
HBC – hereditary breast cancer syndromes
HBOC – hereditary breast and ovarian cancer syndrome
CNV – copy number variation
Array-CGH – comparative genomic hybridization on microarrays
MLPA – multiplex ligation probe amplification NCCN – National Comprehensive Cancer Network IARC - International Agency for Research on Cancer UCSC – University of California, Santa Cruz

Competing interests
The authors declare that they have no competing interests.

Authors' contribution
AG carried out the molecular genetics studies and has drafted the manuscript. IPE carried out part of the MLPA assays and characterized the rearrangement breakpoints by sequencing.

MS carried out part of the MLPA assays of the TP53 gene.
MP characterized the rearrangement breakpoints by sequencing. AP characterized the rearrangement breakpoints by sequencing. AFN participated of the clinical trial and classification of the families.
DMC participated in the design of the study and revised the manuscript.

MIWA and PAP were physicians responsible for the clinical trial, selection and classification of the families, and critically revised the manuscript.
PAP and MT supervised the BRCA1 MLPA and sequencing analyses and result interpretation.
CR revised critically the manuscript.
ACVK participated in the design of the study, performed part of the molecular genetics analysis, and helped to draft the manuscript.

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Links

National Comprehensive Cancer Network [http://www.nccn.org/] Database of Genomic Variants [http://projects.tcag.ca/variation/] International Agency for Research on Cancer [http://www.iarc.fr/] UCSC Genome Bioinformatics [http://genome.ucsc.edu/]

Table 1. Characteristics of the probands: clinical phenotype, type of tumor and age of diagnosis (years).

Figure 1. Mapping of the intragenic BRCA1 deletion detected in a patient with multiple primary tumors and a cancer family history fulfilling criteria for TP53 and BRCA testing. In the upper panel, the array-CGH profile of a region at chromosome band 17q21.31, showing a heterozygous loss in copy number (red bar) of a genomic segment (image adapted from the Genomic Workbench software, Agilent Technologies). The lower panel displays the deleted segment (solid black bar) in the context of the genomic region, encompassing exons 9-19 of the BRCA1 gene according to the analysis of breakpoint sequencing data (figure adapted from UCSC Genome Bioinformatics, http://genome.ucsc.edu, Build 37.1).

Figure 2. Breakpoint sequencing analysis. Eletropherogram showing the g.29197_65577del36381 mutation in the BRCA1 sequence; the intron 8 sequence is followed by intron 19 sequence. The blue arrow represents the inferred breakpoint.

Figure 3. Pedigree of the family with a large BRCA1 rearrangement. Type of cancer is indicated under the subjects and the age of diagnosis is shown in brackets.

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Table1. Characteristics of theprobands: clinical phenotype,typeof tumor and age of diagnosis(years).

Individual Code	Classification	Breast tumor (age at diagnosis)	Other tumors (age at diagnosis)
Y0006T000	Birch	breast (79)	Lymphoma (73), skin (81)
Y29T000	Birch/HBC	breast (26)	Osteosarcoma (19), soft tissue sarcoma (23), head/neck (24)
Y36T000	Birch/HBC	breast (44)	
Y41T000	Chompret/HBC	breast (28)	Osteosarcoma (8)
Y51T000	Eeles1	breast (53)	
Y54T000	Eeles1/HBC	breast (41)	Endometrium (44)
Y83T000	Chompret/HBC	breast (45)	Soft tissue sarcoma (21)
Y93T000	LFS/HBC	breast (42)	
Y95T000	Eeles2/HBC	breast (36)	
Y101T000	Eeles1/HBC	breast (48)	Thyroid (52)
Y110T000	Eeles1/HBC	breast (36)	
Y112T000	Chompret/HBOC	breast (34)	
Y115T000	Chompret/HBC	breast (36)	
Y116T000	Eeles2/HBC	breast (48)	
Y117T000	Eeles1/HBC	breast (44)	
Y122T000	Eeles1/HBC	breast (61)	Colorectal cancer(68)
Y123T000	Eeles1/HBC	breast bilateral (37)	
Y126T000	Chompret/HBC	breast (39)	Lymphoma (23), skin (40)
Y135T000	Eeles1/HBC	breast (30)	
Y143T000	Eeles1/HBC	breast (?)	
Y145T000	Chompret/HBC	Breast, bilateral (36;36)	
Y147T000	Chompret/HBC	breast (35)	Melanoma (36)
Y152T000	LFS/HBC	breast (38)	Skin (36)

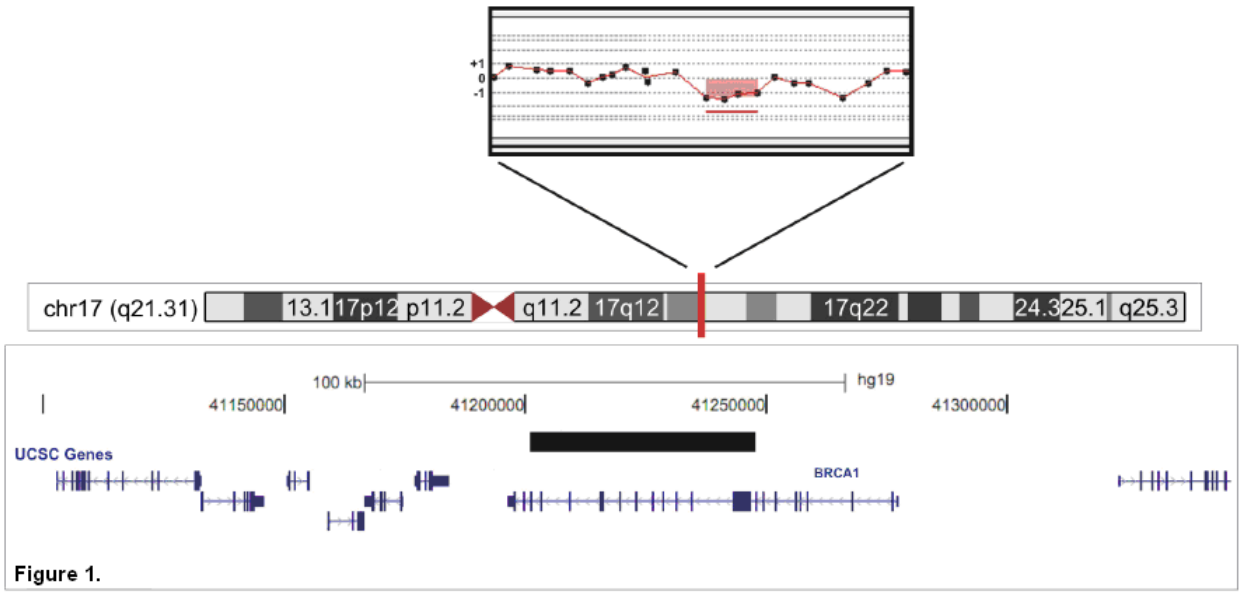

Figure 1.

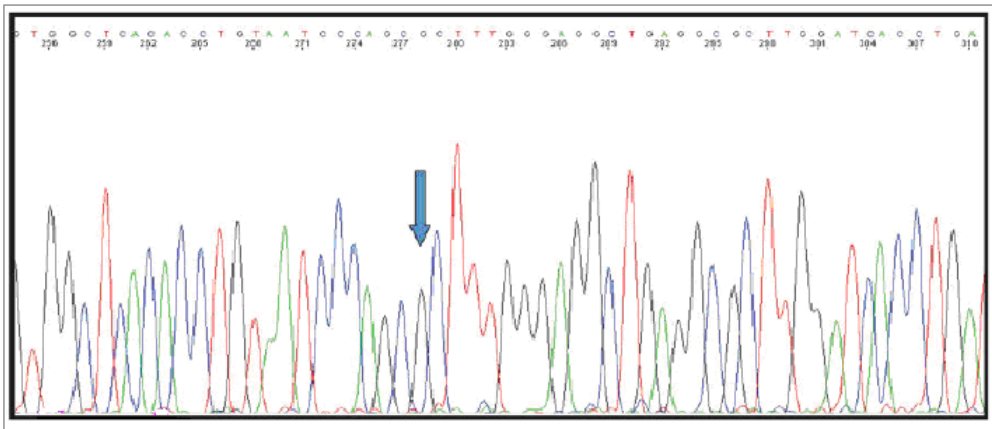

Figure 2.

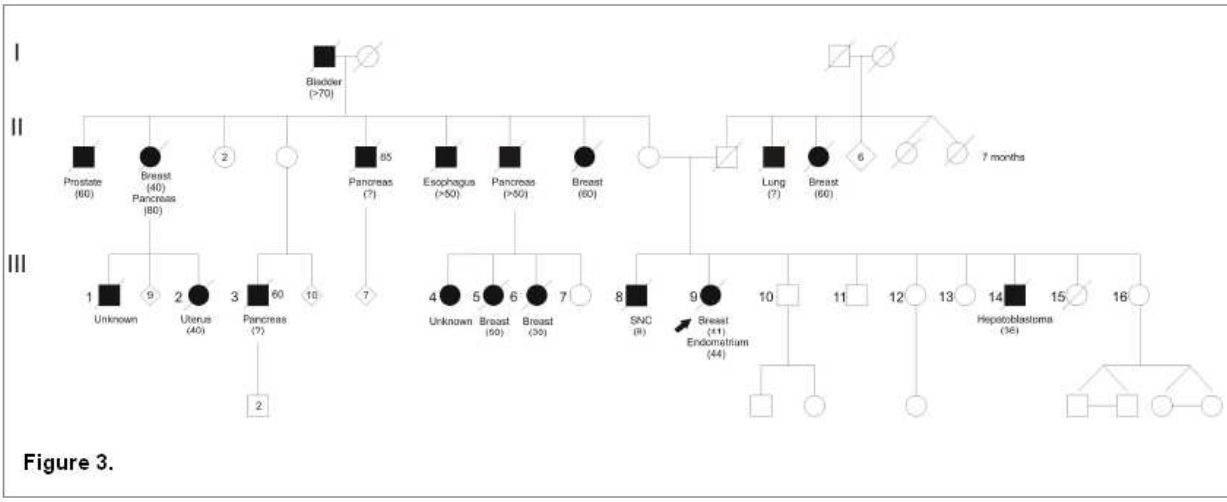

Figure 3.

8. MANUSCRITO 4: *BRCA1* and *BRCA2* rearrangements in Brazilian individuals with the Hereditary Breast and Ovarian Câncer Syndrome.

BRCA1 and *BRCA2* rearrangements in Brazilian individuals with Hereditary Breast and Ovarian Cancer Syndrome

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Keywords: Breast cancer, Hereditary Breast and Ovarian Cancer syndrome, gene rearrangements, *BRCA* gene.

ABSTRACT

Breast cancer is one of the most common malignancies affecting women worldwide. It is well known that approximately 5-10% of the diagnoses are caused by germline mutations in high penetrance predisposition genes. Among these, *BRCA1* and *BRCA2*, associated with Hereditary Breast and Ovarian Cancer (HBOC) syndrome, are the most frequently affected genes. The observed frequencies of *BRCA1/2* mutations in HBOC families are lower than predicted by linkage analysis. Recent studies confirm that gene rearrangements, especially in *BRCA1,* are responsible for a significant proportion of mutations in certain populations. In this study, we determined the prevalence of *BRCA* rearrangements in 145 unrelated Brazilian individuals at-risk for HBOC syndrome that were not previously tested for *BRCA* mutations. Using Multiplex Ligation-dependent Probe Amplification (MLPA) and a specfic PCR-based protocol to identify a Portuguese founder *BRCA2* mutation we identified 4 (2,75%) individuals with germline *BRCA1* mutations (including a deletion in exon 19 (two patients) and a deletion in exons 9-19 and exons 16-17) and 3 probands with the c.156_157insAlu founder *BRCA2* rearrangement. This study comprises the largest Brazilian series of HBOC families tested for *BRCA1* and *BRCA2* rearrangements to date, and includes patients from three regions of the country. The overall observed rearrangement frequency of 3,44% indicates that rearrangements are relatively uncommon in the admixed population of Brazil.

INTRODUCTION

Approximately 5-10% of all breast cancer diagnoses are associated to germline mutations in high penetrance cancer predisposition genes. Among these, the tumor suppressor genes *BRCA1*(OMIM # 113705)and *BRCA2* (OMIM # 600185) are the most frequently affected and better studied. Presence of a germline mutation in these genes defines the Hereditary Breast and Ovarian Cancer (HBOC) syndrome, an autosomal dominant disorder, that predisposes affected individuals to several early-onset tumors including breast, ovarian, prostate, pancreatic cancer and melanoma. Identification of at-risk individuals is important since several risk-reducing strategies can be offered to at-risk patients, especially if they are not yet affected by cancer [1-5].

Hundreds of deleterious germline *BRCA1* and *BRCA2* mutations have been described in all populations and most frequently are single base substitutions (predominantly nonsense mutations) or small frameshift insertions/deletions, which result in premature stop codons and truncated non-functional proteins (http://research.nhgri.nih.gov/bic/) [6, 7,11]. However, in many studies, the observed frequencies of deleterious *BRCA1* and *BRCA2* mutations in HBOC families are lower than predicted by linkage analysis or mutation probability models: pathogenic variations in the coding region or in splice sites of the genes are found in, at most, two-thirds of *BRCA*-mutations-carrier families [8,9]. Several explanations for this observation have been proposed, including heterogeneous inclusion criteria with different stringencies, existence of other dominant genes associated with the phenotype and/or additive effect of multiple lower penetrance alleles. In addition, presence of pathogenic alterations that escape most of the current gene sequencing-based diagnostic approaches were proposed, including partial or complete exon losses or duplications resulting in an out-of-frame translation and in a mutant peptide with abnormal structure and/or function [10,11]. Several reports confirmed indeed that *BRCA* gene rearrangements, particularly in *BRCA1,* are quite frequent in HBOC families from selected countries [12-16]. These mutations are scattered throughout the gene and although most of them are deletions, duplications and triplications, and combined deletion/insertion events have also been described. The higher prevalence of rearrangements in *BRCA1,* as compared to *BRCA2,* has been attributed to its molecular structure, characterized by an extremely high density of intronic *Alu* repeats and by the presence of a duplicated promoter region containing a pseudogene that favors unequal homologous recombination events. [17-20].

The highest proportion of *BRCA1* rearrangements in HBOC families has been observed in The Netherlands and represent approximately 36% of the identifiable mutations

in the gene in this population [21,22]. A similar frequency of deleterious *BRCA1* gene rearrangements has been described in HBOC families from Northern Italy [23] and a recent study of Portuguese HBOC families identified a single founder *BRCA2* rearrangement (c. 156_157insAlu) in 8% of the families studied [19,24-27]. In contrast, Danish families with HBOC have a *BRCA1* rearrangement prevalence less than 5%, in Finland and Canada few or no *BRCA1* rearrangements have been identified in high-risk families. Considering the specificity of mutation prevalence in different populations, and the importance of the precise identification of mutation carriers in at-risk families, the aim of this study is to determine the frequency and nature of germline *BRCA1* and *BRCA2* rearrangements in Brazilian HBOC families.

PATIENTS AND METHODS

Patient recruitment

A consecutive sample of 145 unrelated Brazilian patients, diagnosed with cancer and with a significant personal and/or family history of HBOC syndrome, was evaluated at cancer genetic counseling services from three Brazilian Institutions in the Southern (Hospital de Clínicas de Porto Alegre, Porto Alegre – RS; n=69), Southeastern (Brazilian National Cancer Institute – INCa, Rio de Janeiro – RJ; n=43) and Northeastern (Laboratory of Molecular Biology and Oncogenetics - Federal University of Bahia, Salvador – BA; n=33) regions of the country. The 69 probands from Porto Alegre had been previously studied for the Portuguese founder rearrangement c.156_157insAlu in *BRCA2* (Peixoto et al., 2010).

Cancer-affected probands were approached during their routine clinical visits and invited to participate in the study. None of them had been previously tested for germline *BRCA* mutations. All participants signed informed consent, and fulfilled one or more of the following criteria: (a) personal and family history consistent with the American Society of Clinical Oncology (ASCO) criteria for the HBOC syndrome (ASCO Subcommittee on Genetic Testing for Cancer Susceptibility)[28]; or (b) a prior probability for a *BRCA* mutation ≥ 20% using either mutation prevalence tables published by Myriad Genetics Laboratories, Inc. (http://www.myriad.com) or the Penn II mutation prediction model [29-31]. Ethical approval for this study was obtained from the institutional ethics committees of all participating centers.

Detection of *BRCA* rearrangements by MLPA

Relative quantification of the copy numbers of all 24 *BRCA1* and 27 *BRCA2* exons, was performed using the SALSA P002B *BRCA1* and SALSA P045 *BRCA2* MLPA probe mix assay (MRC-Holland, Amsterdam, The Netherlands) as recommended by the manufacturer (MRC-Holland, http://www.mrc-holland.com/). Multiplex-PCR-amplified products obtained with both SALSA MLPA P002B and P045 kits, were separated by capillary gel electrophoresis in an ABI PRISM 3130XL Genetic Analyser and analysed using GeneMapper ID V3.2 Software. Information on copy number was extracted with the Coffalyser V9.4 Software (MRC-Holland, http://www.mrc-holland.com/). All analyses were performed in duplicates and in at least two independent experiments. Positive results were confirmed in an additional independent experiment performed on a second blood sample. Samples showing BRCA1 rearrangements identified by the SALSA MLPA P002B kit were then analyzed by a different set of MLPA probes (SALSA P087 MLPA probemix, MRC-Holland, Amsterdam, The Netherlands).

Characterization of rearrangementbreakpoints

To confirm BRCA rearrangements detected by MLPA, all rearrangement-positive samples were submitted to long range PCR amplification using AmpliTaq Gold® DNA Polymerase (Applied Biosystems, Foster City, USA) and primers specifically designed to the regions of interest(described in Supplemental Materials).Amplification products of long-range PCRwere separated in a 2.0% agarose gel electrophoresis, visualized under UV and the mutant (variant size) amplification products were extracted and purified using the Gel Band Purification Kit (Illustra, GE Healthcare UK limited, Buckinghamshire, United Kingdom) as described by the manufacturer. Isolated PCR-fragments were submitted to bidirectional sequencing using the Big Dye V3.1 Terminator Kit (Applied Biosystems, Forster City, CA, USA) on an ABI Prism 310 Genetic Analyser (Applied BioSystems, Foster City, USA) with standard protocols. All sequencing electropherograms were analyzed using GeneMapper® Software (Applied BioSystems, Foster City, USA).

Detection of the c.156_157insAlu-*BRCA2* mutation by PCR

To identify thec.156_157insAlu mutation, we performed a PCR reaction to amplify exon 3 of *BRCA2* gene. PCR products of were then visualized by electrophoresis. Normal samples showed one band of ~200 pb, and Alu insertion-positive samples showed and extra band of ~550pb. To confirm the presence of the insertion detected in the first PCR, a second PCR with specific primers flanking the Alu insertion were performed. As expected, a band of ~350pb were visualized by electrophoresis. All Alu insertion-positive samples were submitted to sequencing analysis (protocol previously described by Teulges *et al*., 2005) to confirm the presence of this specific rearrangement.

Statistical Analyses

Sample size for this study was estimated using WINPEPI (PEPI-for-Windows), SPSS version 18.0 was used for data handling and statistical analyses. For descriptive analysis, categorical variables were described by their absolute frequencies and quantitative variables were expressed as mean and standard deviation (SD); a significance level of 0.05 was considered acceptable.

Results

Clinical data of the 145 unrelated probands included in this study are summarized in Table 1.The mean age at diagnosis of the first HBOC-associated tumor was 43 years and the most frequent tumor was breast cancer, as expected. Among all probands included, 118 (81,4%) were diagnosed with their first primary tumor before the age of 50 years. The estimated prior probability of carrying a *BRCA* gene mutation was greater than 20% for 65 (44,8%) and 71 (49,0%) probands according to the Myriad prevalence tables and the Penn II model, respectively, and as expected, a larger proportion of families met the less stringent ASCO criteria. *BRCA* mutations were identified in 7 probands (4,82%), being three of them positive for the Portuguese *BRCA2* founder rearrangement c.156_157insAlu. All *BRCA1*- and one of the *BRCA2*-positive probands had multiple primary tumors. Sequencing analyses identified the break-points of two rearrangements identified by MLPA in *BRCA1.* The first case (proband 24) had a microdeletion comprising exons 9 to 19, visualized after long-range PCR amplification of the flanking regions as a variant amplification product of approximately 450 bp, when compared to the wild-type allele amplification product of 9 kb. Bidirectional sequencing of the variant allele identified the exact breakpoints and characterized this

rearrangement as g.29197_65577del36381 (Figure 1A). The second case (proband 117) had a microdeletion comprising exons 16 and 17, visualized after long-range PCR amplification of the flanking regions as a variant amplification product of approximately 590 bp when compared to the wild-type allele amplification product of 6 kb. Bidirectional sequencing of the variant allele identified the exact breakpoints and characterized this rearrangement as c.4675+467_5075-990del(Figure 1B). In the other two cases (proband 26 and proband 32), with a suspected deletion of exon 19, confirmatory MLPA with a second set of probes failed to confirm the presence of a rearrangement and further sequencing of the region identified the frameshift 5296del4 founder mutation initially described in African Americans (NM_007294.2: c.5177_5180delGAAA) (Figure 1C), which is localized within the sequence corresponding to one of the *BRCA1* exon 19 probes of the SALSA P002B *BRCA1* set(Figure 1). Description of the clinical and family history features of the four germline *BRCA1* and of the three *BRCA2*mutation carriers identified is summarized in Table 2

DISCUSSION

Using MLPA as a first approach to identify *BRCA1/BRCA2* germline mutations in a sample of cancer-affected Brazilian individuals with a high clinical suspicion for HBOC syndrome, we identified four patients (2,75%) with germline BRCA mutations, and two of them were confirmed to be large rearrangements.Additional screening with a PCR-based method to identify a Portuguese founder BRCA2 rearrangement identified three other mutation positive families. *BRCA1* rearrangements are more prevalent than those in *BRCA2*mostly due to the high density of *Alu* elements throughout the *BRCA1* locus, which seem to be particularly frequent in certain populations. In addition to possible founder effects in specific populations, rearrangements have been most commonly encountered in probands and families with multiple primary cancer diagnoses in at least one individual [32-37]. This phenotype was also observed in the majority of rearrangement-positive patients from the present series, reinforcing that rearrangements should always sought for in families where at least one cancer-affected individual has more than one primary tumor.

The *BRCA1* deletion 9-19, identified in a proband that developed breast cancer at the age of 41 and endometrial cancer at the age of 44 years, and had a significant cancer history with the presence of several early-onset *BRCA*-related tumors is the same as the one identified in an Italian patient (Montagna et al, 2003). We were able to trace family history back to the proband´s maternal grandfather, who emmigrated to Brazil from Italy in the 19th century. On the other hand, deletions involving exons 16 or 17, are quite common and have

been described in several populations. However, a rearrangement involving breakpoints at Alu regions in intron 15 and in intron 17 had not been previously described [42-44]. One of the most interesting results from this mutation screening strategy is the identification of a small frameshift mutation (a deletion of four nucleotides in *BRCA1* exon 19, 5296del4) in two families by MLPA. Since the mutation occurs within the sequence of the MLPA probe for exon 19, hybridization did not occur and a call for an exon 19 deletion was made. The use of a second MLPA kit, with a different probe for that specific region failed to identify a rearrangement and sequencing through the region confirmed the frameshift mutation. This illustrates the importance of always confirming results of MLPA, which is considered a screening strategy with an alternative mutation detection method in the diagnostic setting. Interestingly, this particular frameshift mutation has been previously described as a founder mutation in African Americans and has been associated with more aggressive tumors, diagnosed at younger ages. Both of the mutation-positive families identified in our study reported European ancestry (German), and although the probands have been diagnosed with multiple primaries, there is no evidence in either of them for a more aggressive clinical course. [38-41].

Considering the existence of a founder BRCA2 rearrangement (c.156_157insAlu), which is very common in Northern Portugal, and is not identifiable by either sequencing or MLPA, we added a second screening protocol, specific for the mutation, in this investigation. This enabled identification of the founder in three families which is not unexpected given the high proportion of Portuguese descendants among the Brazilian population (Marrero *et al.*, 2005). Our results reinforce the importance of characterizing mutations in specific populations.

Most of the studies describing the prevalence of *BRCA1* rearrangements in HBOC individuals have screened for such mutations only after a negative result in full gene sequencing. Considering the cost and complexity of sequencing the entire coding region of both *BRCA1* and *BRCA2* genes, we designed this study to verify whether MLPA and a specific protocol for a founder BRCA2 mutation could be cost-effective strategies as a first mutation screening approach. Although these screening strategies were effective in identifying germline *BRCA1* mutations and the Portuguese founder mutation in this series, mutation frequency was relatively low and in the majority of the patients included, molecular diagnosis remains undetermined. Thus, we conclude that MLPA can be used as an initial approach for the diagnosis of *BRCA1* mutations in HBOC families especially considering that it is an unexpensive and straightforward methodology which enables mutation screening of the entire coding region of a gene in a few hours. In populations where a known founder mutation occurs, screening for this specific founder as an initial step is also acceptable.

However, continued investigation by gene sequencing must be proposed in all risk families when these initial approaches have negative results. This study comprises the largest Brazilian series of HBOC families tested for *BRCA1* and *BRCA2* rearrangements to date, and includes patients from three regions of the country. The overall rearrangement frequency observed of less than 5% indicates that rearrangements are relatively uncommon in this country.

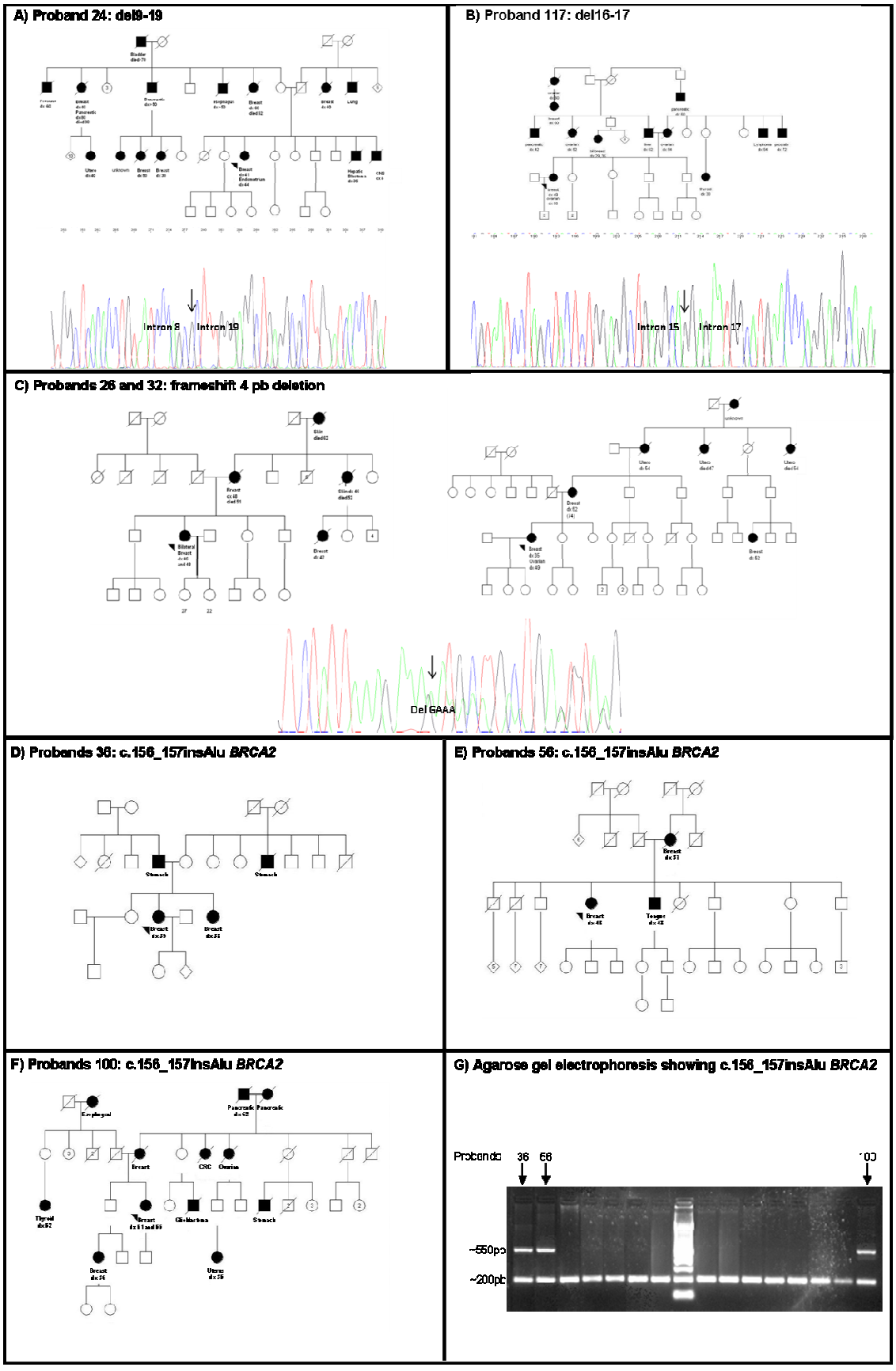

Figure 1. Pedigrees and molecular results of the mutation-positive probands. Panels A, B and C: families with germline *BRCA1* mutations identified by MLPA as first mutation screening strategy. Panels D, E

and F: families with germline c.156_157insAlu-*BRCA2* mutation identified by PCR. Panel G: agarose gel electrophoresis showing the presence of an aberrant band (~550pb) corresponding to the Alu insertion in probands 36, 56 and 100.

Table 1 – Clinical characterization of the series (n=145) of HBOC patients included in this study

Feature	N	%	Mean (SD) in years
Sex			
Female	144	99,9	
Age at breast cancer (years)			42.92 (9.5) range: 19-69
Breast cancer diagnosed< 50 years	118	81,4	
HBOC syndrome criteria[1]			
ASCO	84	57,9	
Mutation prevalence[2] (Myriad) ≥ 20%	65	44,8	
Prior probability[3] (Penn II) ≥ 20%	71	49,0	
Bilateral breast cancer	18	12,4	
Type of tumor in the proband			
Breast cancer	129	88,9	
Ovarian cancer	6	4,2	
Colorectal cancer	4	2,8	
Other[4]	6	4,1	
Multiple primaries	31	21,4	
≥2 Breast	14		
1 breast and 1 ovarian	6		
≥2 Breast and 1 ovarian	3		
At least one breast + other	7		
At least one ovarian + other	1		

1. One proband may fulfill more than one criterion
2. Patients with a family history compatible with mutation prevalence of ≥ 20%
3. Estimated prior probability of being a germline BRCA mutation carrier
4. Gastric cancer, melanoma, carcinoma of the uterine cervix, prostate and kidney cancer.

Table 2. Clinical features of the seven probands with identifiable germline *BRCA1* mutations.

Case #	*BRCA1/BRCA2* mutation identified	Cancer diagnosis (index-case)	Age at diagnosis of first primary (years)	Cancer family history*	ASCO criteria	Prior Probability of Mutation in *BRCA*	
						Mutation prevalence (Myriad) (%)	Prior probability of mutation (Penn II) (%)
24-RS	Deletion exons 9-19: g.29197_65577 del36381	Multiple primary: breast and endometrial	41	MAT Hepatob (M-36), Esoph (M-N/A), Br (F-30), Panc (M-N/A), Blad (M-N/A), Br (F-50), Br and Panc (F-40,80), Prost (M-60), Ut (F-40), CNS (M-8), Br (F-60). PAT Br (F-60), Lu (M-N/A)	Yes No	30,1 6,9	36,0 10,0
117-BA	Deletion exons 16-17 c.4675+467_5075 -990del	Multiple primary: breast and ovarian	49	MAT Ovarian (F-54), Thyr (F-30), Lymph (M - 54) and Prost (M-72). PAT Liv = (M 62), Ovarian (F52), Ovarian (F 60), Panc (M-42), Bilateral Br (F-29,35), Br (F 60), Panc (M 60).	Yes	79,0	90,0
32-RS	5296del4	Multiple primary: breast and ovarian	35	MAT Br (F-52), Ut (F-54), Ut (F-47) Ut (F-N/I), Br (F-52)	Yes	39,1	29,0
36-RS	5296del4	Multiple primary: bilateral Breast	46	MAT Br (F-48), Br (F-42),Skin (F-46), Skin (F-N/A)	Yes	30,1	18,0
100-RS	c.156_157insAlu	Multiple primary: bilateral Breast	51	MAT Br (F-62), CCR (M-80), Ut (F-35), Ovarian (F-45), stomach(M-52),panc (M-62), panc (F- 67) , Glioblast (M-38)	Yes	10,6	40,0
36-RJ	c.156_157insAlu	Breast	39	MAT Br (F-36), Stomach (M-N/A)	Yes	15,8	14,0
56-RJ	c.156_157insAlu	Breast	48	MAT Br (F-37), Tongue (M-45),	Yes	15,8	14,0

Legend: RS = family recruited from Rio Grande do Sul; BA = family recruited from Salvador – BA.MAT = cancer history in the maternal side of the family, PAT = cancer history in the paternal side of the family; * Other cancer diagnoses in family are indicated by the abbreviated cancer type (Br = breast, Prost = prostate; Esoph = esophageal; Hepatob = hepatoblastoma; End= endometrial; CNS = central nervous system, panc = pancreatic, blad = bladder; Thyr =Thyroid; Lymph = Lymphoma; Glioblast = glioblastoma, Ut = uterine cancer, not defined whether cervix or endometrium) followed by sex (M = male, F = female) and age at diagnosis (N/A= not available).

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SUPPLEMENTARY MATERIALS

Primers	Nucleotide sequence
Deletion comprising exon 9 to exon 19, *BRCA1* gene	5'-ACTCTGAGGACAAAGCAGCGGA-3' 5'-GTGCCACCAAGCCCGGCTAA-3'
Deletion comprising exon 16 and 17, *BRCA1* gene	5-'TGAGGATGAGGGAGTCTTGGTGTAC-3' 5'-TCCTAAACACAGCAGGCTATCTGCA-3'
Deletion of exon 19 of *BRCA1* gene	5'-TCTATCTCCGTGAAAAGAG-3' 5'-CTGGTTAGTTTGTAACATC-3'
Exon 3 of *BRCA2*	5'-GTC ACT GGT TAA AAC TAA GGT GGG A-3' 5'-GAA GCC AGC TGA TTA TAA GAT GGT t-3'
Alu insertion in *BRCA2*: c.156_157insAlu	5'-GAC ACC ATC CCG GCT GAA A -3' 5'- CCC CAG TCT ACC ATA TTG CAT-3'

9. CONSIDERAÇÕES FINAIS E PERSPECTIVAS

O presente estudo teve como objetivo verificar a frequência de rearranjos gênicos em *BRCA1* e *BRCA2* em indivíduos brasileiros em risco para a Síndrome de câncer de mama e ovário hereditários (HBOC). Tradicionalmente, o diagnóstico molecular dessa síndrome é particularmente difícil, pois os genes associados, *BRCA1* e *BRCA2,* são genes grandes (82.9Kb e 86.1Kb, respectivamente) e há grande heterogeneidade molecular, tendo sido identificadas centenas de mutações patogênicasdistribuídas ao longo de toda a seqüência codificadora e de diferentes tipos, sendo as mutações de ponto ou pequenas inserções ou deleções as mais comuns. Em certas populações fora do Brasil ou mesmo em amostras de famílias Brasileiras com HBOC, foram descritas mutações fundadoras que são significativamente mais prevalentes possibilitando uma abordagem inicial de rastreamento simplicado.

Nesta tese, e em amostras de pacientes em risco para HBOC atendidas em ambulatórios de risco para câncer hereditário nos estados do Rio de Janeiro (região Centro-oeste), Bahia (região Nordeste) e Rio Grande do Sul (região Sul), os três estados Brasileiros com as mais altas taxas de incidência de câncer de mama no país, foram realizados dois estudos de frequencia de rearranjos em genes *BRCA*. Em um primeiro estudo a mutação fundadora Portuguesa c.156_157*insAlu* em *BRCA2*, freqüente em famílias HBOC do Norte dePortugal, (Machado *et al.*, 2007; Peixoto *et al*., 2009; Teugels *et al*., 2005) foi rastreada em múltiplos países da Europa, América do Norte, América do Sul e Ásia. Nosso laboratório contribuiu com98 casos-índice não-relacionados, com fenótipo de HBOC e procedentes de Porto Alegre, Rio Grande do Sul, não sendo identificado nenhum indivíduo portador

da mutação fundadora c.156_157*insAlu*. Adicionalmente, colaboradores brasileiros do Estado de São Paulo incluíram na amostra estudada mais 46 casos não tendo igualmente identificado portadores (total de Brasileiros avaliados = 144). Nessa primeira abordagem, portanto, a inexistência de pacientes portadores em uma amostra de pacientes em risco, indicava baixa prevalência da mutação fundadora c.156_157insAlu em indivíduos Brasileiros, ao contrário do esperado pela significativa contribuição de Portugueses para o pool genético da população Brasileira. No entanto, informação recente de colaboradores do Estado do Rio de Janeiro (Vargas FR, comunicação pessoal 2011) confirmava a presença da mutação fundadora Portuguesa em um pequeno número de pacientes daquele Estado. Considerando que a prevalência poderia ser distinta em diferentes regiões Brasileiras de acordo com a contribuição diferencial de alelos Portugueses (especialmente do Norte de Portugal) em diferentes regiões do País, decidimos ampliar a nossa amostra de estudo, o que nos estimulou a realização do segundo estudo apresentado aqui.

No segundo estudo, propusemos o rastreamento inicial de pacientes com a síndrome HBOC utilizando uma combinação de estratégias para identificação de rearranjos: MLPA para BRCA1 e BRCA2 associados ao mesmo protocolo baseado em PCR para a detecção do rearranjo fundador de BRCA2. Essa estratégia foi escolhida pela sua simplicidade e baixo custo. Em 145 casos-índice com critérios clínicos de HBOC foi realizado o rastreamento para rearranjos gênicos antes de qualquer outra investigação molecular, sendo identificados 4 (2,7%) portadores de mutação germinativa no gene *BRCA1*(sendo 2 rearranjos e 2 mutações de mudança da matriz de leitura) e 3 (2.1%) portadores do rearranjo fundador Português em*BRCA2.* Embora o resultado do rastreamento de rearranjos gênicos em *BRCA1 e*

BRCA2 aqui descritos indique uma baixa frequencia na amostra global, os resultados obtidos por esta estratégia de rastreamento inicial, especialmente considerando baixo custo e simplicidade metodológica, podem justificar o seu uso como abordagem inicial em indivíduos Brasileiros com HBOC. Entretanto, devido a grande heterogeneidade molecular da síndrome, nos casos em que os resultados do rastreamento inicial são negativos, é imprescindível analisar toda a seqüência codificadora dos genes *BRCA1* e *BRCA2* por sequenciamento bidirecional. Estudos adicionais, incluindo rastreamento de mutações pontuais deletérias nos genes *BRCA1* e *BRCA2*, fazem-se necessários para um rastreamento molecular mais eficaz e informativo para finalidade de aconselhamento genético. O entendimento da sensibilidade e especificidade de cada um dos métodos de rastreamento e diagnóstico de mutações nos genes *BRCA* é essencial para a realização de um adequado aconselhamento genético e para a definição mais objetiva do risco em famílias afetadas.

10. CONCLUSÕES

1. Afrequência de rearranjos gênicos no gene *BRCA1* identificados por MLPA (*Multiplex Ligation-dependent Probe Amplification* – MLPA) em uma estratégia de primeira abordagem de rastreamentomolecular foi de 2 em 145 casos analisados (1,4%), não tendo sido observados rearranjos de *BRCA2* identificáveis por essa técnica. Em ambos os casos o rearranjo identificado envolvia sequencias Alu intrônicas. Um dos rearranjos (deleção dos exons 9-19 de *BRCA1*) havia sido descrito anteriormente, e o outro, (deleção dos exons 16-17de *BRCA1)*, foi descrito pela primeira vez neste estudo. A baixa frequência de resultados positivos impede conclusão acerca da existência de um rearranjo fundador Brasileiro, embora a

amostra estudada não possa permitir identificação de uma alteração fundadora mais rara.

2. A freqüência da mutação fundadora Portuguesa c.156_157ins*Alu* no exon 3 de*BRCA2* em famílias Brasileiras com HBOC em uma estratégia de primeira abordagem de rastreamentomolecular foi de 3 em 145 casos estudados (2.1%).

3. As duas famílias com rearranjos em *BRCA1* são famílias com múltiplos tumores, reforçando que rearranjos gênicos, aparentemente são mais freqüentes em famílias com múltiplos tumores, o que poderia justificar a indicação inicial de triagem para rearranjos quando há este fenótipo.

4. As três famílias com rearranjo fundador Português em BRCA2, são famílias com diagnósticos de câncer de mama em idade jovem e aparentemente não são descendentes diretos de Portugueses. No entanto, se considerarmos o baixo custo e a simplicidade metodológica da técnica podem justificar o seu uso como abordagem inicial em indivíduos Brasileiros com HBOC.

As duas famílias com a mutação frameshift identificada, são famílias não relacionadas e possuem a mesma mutação fundadora Afro Americana Aka 5296del4, com diagnóstico de múltiplos tumores, levando também a uma associação com a possível indicação inicial de triagem para este fenótipo.

Este estudo compreende a maior série brasileira de famílias HBOC testadas rearranjos gênicos em *BRCA1* e *BRCA2* até o momento e inclui pacientes de três regiões do país. A frequência de rearranjos global observada de menos de 5% indica que rearranjos são relativamente raros neste país.

11. ANEXOS

11.1 Produção científica adicional no período (resumos)

10.1.1 FREQÜÊNCIA DA MUTAÇÃO 5382insC NO GENE *BRCA1* EM UM GRUPO DE PACIENTES BRASILEIRAS COM CÂNCER DE MAMA BILATERAL

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As síndromes genéticas de predisposição ao câncer de mama (CM) estão associadas a mutações germinativas em genes supressores de tumor de alta penetrância. A síndrome de predisposição hereditária ao câncer de mama mais importante em número relativo de casos é a chamada síndrome de HBOC (do inglês *Hereditary Breast and Ovarian Cancer Syndrome:* Síndrome de Câncer de Mama e Ovário Hereditário), causada principalmente por mutações germinativas nos genes *BRCA1* e *BRCA2*. A mutação germinativa 5382insC no gene *BRCA1*tem sido encontrada em pacientes com câncer de mama bilateral, bem como em pacientes com diagnóstico de CM em idade precoce. Sendo assim, este estudo pretende estimar a freqüência da mutação 5382insC em 80 pacientes não relacionados de alto risco para a Síndrome de HBOC com câncer de mama bilateral provenientes de três estados Brasileiros (Rio Grande do Sul, São Paulo e Bahia). A análise da mutação germinativa 5382insC foi realizada por PCR seguido de seqüenciamento em DNA extraído a partir de sangue periférico dos pacientes incluídos. Dos 80 casos analisados, 5% (quatro pacientes) apresentou a mutação.De acordo com esses resultados, o rastreamento da mutação germinativa 5382insC poderia ser utilizado como primeira abordagem em pacientes com diagnóstico de CM bilateral. Identificar pacientes de alto risco portadores de mutação germinativa em genes de alta penetrância é fundamental para o adequado manejo do paciente e da sua família.

10.1.2 FREQUENCIA DA MUTAÇÃO 2152 C>T–MHS2 EM FAMÍLIAS BRASILEIRAS COM SÍNDROME DE LYNCH

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A Síndrome de Lynch (LS), doença autossômica dominante de predisposição ao câncer, é causada por mutações germinativas em um dos prinicpais genes do sistema MMR de reparo do DNA: MLH1, MSH2, MSH6 e PMS2. Clínicamente, a SL se caracteriza pelo desenvolvimento de câncer colorretal em idade precoce, bem como outros tumores extra-colônicos. O diagnóstico clínico da SL é realizado quando a família do indivíduo afetado preenche os critérios de Amsterdam. Critérios menos estritos (critérios de Bethesda) têm sido utilizados para a identificação de indivíduos em risco com história familiar sugestiva. Mutações germinativas em MLH1 e MSH2 são encontradas na maioria (50-90%) das famílias Lynch. . Em 1999, uma mutação nova (c.2152 C>T-MSH2) foi descrita em famílias Lynch portuguesas. Este estudo tem por objetivo estimar a frequencia da mutação c.2152C>T em famílias brasileiras com SL. Para isto, foram incluídos 49 indivíduos não relacionados com diagnóstico clínico da SL. A análise d a mutação germinativa c.2152C>T–MSH2 foi realizada em DNA extraído a partir de sangue periférico, através da técnica de PCR seguida de sequenciamento. Dos pacientes analisados, 6.12% apresentou a mutação. A relativa frequencia dessa mutação na população brasileira sugere a análise da mesma como primeira abordagem no rastreamento molecular em indivíduos de origem portuguesa com diagnóstico clínico da SL.

10.1.3 Prevalência da mutação fundadora c.5266dup no gene *BRCA1* em indivíduos Brasileiros em risco para a Síndrome de Câncer de Mama e Ovário Hereditários

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Prevalence of the *BRCA1* founder mutation c.5266dupin Brazilian individuals at-risk for the hereditary breast and ovarian cancer syndrome

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Abstract

About 5-10% of breast and ovarian carcinomas are hereditary and most of these result from germline mutations in the *BRCA1* and *BRCA2* genes. In women of Ashkenazi Jewish ascendance, up to 30% of breast and ovarian carcinomas may be attributable to mutations in these genes, where 3 founder mutations, c.68_69del (185delAG) and c.5266dup (5382insC) in *BRCA1* and c.5946del (6174delT) in *BRCA2*, are commonly encountered. It has been suggested by some authors that screening for founder mutations should be undertaken in all Brazilian women with breast cancer. Thus, the goal of this study was to determine the prevalence of three founder mutations, commonly identified in Ashkenazi individuals in a sample of non-Ashkenazi cancer-affected Brazilian women with clearly defined risk factors for hereditary breast and ovarian cancer (HBOC) syndrome. Among 137 unrelated Brazilian women from HBOC families, the *BRCA1*c.5266dup mutation was identified in seven individuals (5%). This prevalence is similar to that encountered in non-Ashkenazi HBOC families in other populations. However, among patients with bilateral breast cancer, the frequency of c.5266dup was significantly higher when compared to patients with unilateral breast tumors (12.1% vs 1.2%, $p = 0.023$). The *BRCA1* c.68_69del and *BRCA2* c.5946del mutations did not occur in this sample. We conclude that screening non-Ashkenazi breast cancer-affected women from the ethnically heterogeneous Brazilian populations for the *BRCA1* c.68_69del and *BRCA2* c.5946del is not justified, and that screening for *BRCA1*c.5266dup should be considered in high risk patients, given its prevalence as a single mutation. In high-risk patients, a negative screening result should always be followed by comprehensive *BRCA* gene testing. The finding of a significantly higher frequency of *BRCA1* c.5266dup in women with bilateral breast cancer, as well as existence of other as yet unidentified founder mutations in this population, should be further assessed in a larger well characterized high-risk cohort.

Keywords: Hereditary breast cancer, Hereditary breast and ovarian cancer Syndrome, Founder mutations, *BRCA1* gene, *BRCA2* gene

11.2 Critérios de Inclusãoe instrumentos para estimativa da probabilidade de mutação em genes BRCA

11.2.1 Tabelas de prevalência de mutação dos Laboratórios Myriad

1. The Prevalence of Deleterious Mutations in BRCA1 and BRCA2 (Excludes Individuals of Ashkenazi Ancestry)

	Family History (Includes at least one first or second degree relative)					
Patient's History	No breast cancer <50, or ovarian cancer, in any relative.†	Breast cancer <50 in one relative; no ovarian cancer in any relative.	Breast cancer <50 in more than one relative; no ovarian cancer in any relative.	Ovarian cancer at any age in one relative; no breast cancer <50 in any relative.	Ovarian cancer in more than one relative; no breast cancer <50 in any relative.	Breast cancer <50 and ovarian cancer at any age. ††
No breast cancer or ovarian cancer at any age	2.8%	4.5%	8.7%	5.6%	9.6%	12.2%
Breast cancer ≥ 50	2.9%	5.3%	11.4%	6.4%	12.2%	15.9%
Breast cancer <50	6.8%	15.8 %	30.1%	16.9%	27.3%	39.2%
Male breast cancer	12.8%	21.8%	41.9%	20.0%	40.0%*	61.9%
Ovarian cancer at any age, no breast cancer	8.8%	23.1%	42.3%	21.1%	33.2%	46.5%
Breast cancer ≥50 and ovarian cancer at any age	17.6%	26.1%	46.2%	30.3%	46.2%	60.0%
Breast cancer <50 and ovarian cancer at any age	39.1%	53.9%	67.2%	66.0%	70.8%	79.0%

† May include families with breast cancer ≥50 (in women or men).
†† Includes family members with either or both diagnoses.
Number of observations in Table 1 is 4914
*N<20

2. The Prevalence of Deleterious Mutations in BRCA1 and BRCA2 in Individuals of Ashkenazi Ancestry

	Family History (Includes at least one first or second degree relative)					
Patient's History	No breast cancer <50, or ovarian cancer, in any relative.†	Breast cancer <50 in one relative; no ovarian cancer in any relative.	Breast cancer <50 in more than one relative; no ovarian cancer in any relative.	Ovarian cancer at any age in one relative; no breast cancer <50 in any relative.	Ovarian cancer in more than one relative; no breast cancer <50 in any relative.	Breast cancer <50 and ovarian cancer at any age. ††
No breast cancer or ovarian cancer at any age	6.9%	13.7%	19.9%	15.6%	23.6%	27.5%
Breast cancer ≥ 50	4.4%	9.4%	11.3%	15.8%	20.0%	19.9%
Breast cancer <50	12.0%	24.2%	38.3%	38.8%	59.2%	51.4%
Male breast cancer	15.0%	30.8%	0.0% *	40.0% *	100.0% *	70.0% *
Ovarian cancer at any age, no breast cancer	22.2%	37.0%	60.6%	42.0%	43.2%	72.3%
Breast cancer ≥50 and ovarian cancer at any age	29.5%	64.3% *	50.0% *	50.0% *	100.0% *	63.6% *
Breast cancer <50 and ovarian cancer at any age	71.1%	88.9% *	80.0% *	90.9% *	100.0%*	75.0% *

† May include families with breast cancer ≥50 (in women or men).
†† Includes family members with either or both diagnoses.
Number of observations in Table 2 is 15345
*N<20
Table 2 includes individuals that tested for MultiSite3, which may have been for a known mutation in the family

11.2.2 Modelo de avaliação de risco de mutação em *BRCA1* e *BRCA2* – Penn II

The Penn II Risk Model - What is the Penn II Model?

This model can be used to predict the pre-test probability, or prior probability, that a person has a BRCA1 or BRCA2 mutation. In general, individuals with at least a 5-10% chance of having a mutation in either gene are considered good candidates for genetic testing.
This model does not predict breast cancer risk. It focuses only on the chance that an individual has inherited a mutation in BRCA1 or BRCA2.

Instructions for use
1. Please answer the following questions.
2. Information from a single lineage in the family should be used and restricted to three generations.
3. If there is cancer history present on both the maternal and paternal sides, each lineage should be entered separately.
4. Since this model depends on the family history being accurate, attempts should be made to confirm the family history with pathology reports, especially for cases of ovarian cancer

Part A. Select the Side of the Family in Question: Maternal Paternal
Part B. Please provide Following Information: no yes
1. Presence of Ashkenazi Jewish ancestry? (0-100)
2. Number of women in family diagnosed with both breast and ovarian cancer? (0-100)
3. Number of individual women in family diagnosed with ovarian or fallopian tube cancer in the absence of breast cancer? (0-100)
4. Number of breast cancer cases in family diagnosed in individuals under the age of 50? (18-130)
5. What is the age of the youngest breast cancer case? no yes
6. Presence of mother-daughter breast cancer diagnosis in family? (0-100)
7. How many individuals with bilateral breast cancer in family? (0-100)
8. Number of male breast cancer diagnoses in family? no yes
9. Presence of pancreatic cancer in family? (0-100)
10. Number of prostate cancer diagnoses in family?
Part C. Closest Relative with Breast or Ovarian Cancer: Aunt/Uncle First Cousin Grandparent/Grandson/Granddaughter Sibling/Parent/Child The Patient/Proband Unknown
Part D. Patient Information (Optional- for use on report only):
1. Patient's first name
2. Patient's last name
3. Patient's age
4. Clinic location

11.2.3 Critérios de inclusão para Síndromes de Predisposição ao Câncer de Mama: CRITÉRIOS DA ASCO

1. 3 ou mais casos de cancer de mama e 1 ou mais de cancer de ovário em qualquer idade;
2. Mais de 3 casos de cancer de mama com dx antes dos 50 anos;
3. 3 casos de cancer de mama com dx antes dos 50 anos;
4. Pares de irmãs (ou mãe-filha) com 2 dos seguintes antes dos 50 anos:
   4A) 2 casos de câncer de mama
   4B) 2 casos de câncer de ovário
   4C) 1 câncer de mama e 1 câncer de ovário

11.2.4 Critérios de inclusão para Síndromes de Predisposição ao Câncer de Mama e Ovário: CRITÉRIOS NCCN

Caso for familiar (ou seja se a paciente não tem câncer) usar "e/" antes do critério

B1. Ca de mama diagnosticado em idade igual ou inferior aos 40 anos, com ou sem história familiar;
B2. Ca de mama diagnosticado em idade igual ou inferior a 50 anos, com um ou mais familiares com câncer de mama ou um ou mais familiares com ca de ovário;
B3. Ca de mama diagnosticado em qquer idade, com no mínimo dois familiares próximos com ca de ovário em qquer idade ou ca de mama, especialmente se, em no mínimo 1 mulher o dx foi antes dos 50 anos ou se foi bilateral;
B4. Familiar do sexo masculino com ca de mama;
B5. História pessoal de ca de mama e de ovário;
B6. Descendência judaica Ashkenazi e dx do ca de mama em idade inferior a 50 anos, mesmo sem HF de ca ou em qquer idade se houver história de ca de mama ou de ovário em algum familiar.
C1. No mínimo 2 familiares com ca de ovário;
C2. 1 familiar com ca de ovário e, no mínimo, 1 mulher com ca de mama < 50 anos ou ca de mama bilateral;
C3. 1 familiar com ca de ovário e, no mínimo, 2 familiares com ca de mama;
C4. 1 familiar com ca de ovário e, no mínimo 1 homem com ca de mama;
C5. 1 familiar com ca de ovário e, se de descendência judaica Ashkenazi, nenhuma HF adicional é requerida.

D1. 2 casos de ca de mama masculino;
D2. 1 caso de ca de mama masculino e 1 ou mais mulheres com ca de mama ou de ovário;
D3. 1 caso de ca de mama masculino e, se de descendência judaica Ashkenazi, nenhuma HF adicional é requerida.

11.3 Termo de Concentimento Livre e Esclarecido (TCLE)

ANEXO 4 - TCLE

IDENTIFICAÇÃO E ANÁLISE DE ALTERAÇÕES (REARRANJOS) EM GENES DE PREDISPOSIÇÃO HEREDITÁRIA AO CÂNCER DE MAMA E OVÁRIO.

INVESTIGADORES: Ingrid Petroni Ewald (1), Edenir Inêz Palmero (1), Patricia Izetti Lisboa Ribeiro(1), Fernando Regla Vargas (2), Miguel Moreira (2), Maira Caleffi (3), Huander Felipe Andreolla (4), Daniela Dornelles Rosa (4), Patricia Ashton-Prolla (1).

(1) Hospital de Clínicas de Porto Alegre	Tel: (51) 2101-7661
(2) Instituto Nacional do Câncer	Tel: (21) 3233-1469
(3) Hospital Moinhos de Vento	Tel: (51) 3314 - 3696
(4) Hospital Fêmina, Hospital Conceição (GHC)	Tel: (51) 33145291

TERMO DE CONSENTIMENTO INFORMADO

OBJETIVO:

Você está sendo convidado a participar de um projeto de pesquisa que vai estudar alterações genéticas no gene *BRCA* que podem causar maior risco para câncer de mama e de ovário. Estão sendo selecionadas para este estudo famílias que tenham pessoas diagnosticadas com câncer de mama e ovário e que preenchem alguns critérios especiais em termos do número de casos de câncer na família e idade ao diagnóstico de câncer.

PROCEDIMENTOS QUE SERÃO UTILIZADOS:

O estudo envolve pelo menos uma consulta de aconselhamento genético (AG), leitura e assinatura do termo de consentimento, coleta de sangue e teste genético. A sua participação nesse estudo é voluntária e não vai influenciar ou modificar seu acesso a tratamento médico agora ou no futuro. Como esse é um projeto de pesquisa e o exame não será realizado em um laboratório comercial, não há prazo exato nem garantia absoluta de resultado conclusivo (o resultado do teste poderá ser inconclusivo por material insuficiente ou inadequado, por exemplo). Porém, todo esforço será feito para que o resultado seja liberado o mais breve possível. O teste genético requer a coleta de 10 ml de sangue do paciente para estudo de alterações genéticas em genes de predisposição e em algumas vezes, poderá ser necessário repetir essa coleta. Você tem a opção de não querer receber ou retardar o recebimento dos resultados da análise genética durante qualquer momento do processo de testagem.

Se você quiser saber qual é o resultado, este será fornecido durante uma sessão pessoal de aconselhamento genético no Ambulatório de Genética do Câncer do Hospital de Clinicas. A princípio, esse aconselhamento é realizado individualmente, mas você poderá trazer um familiar ou outra pessoa para acompanhar a consulta. O resultado não será transmitido por telefone, fax ou carta. Durante o aconselhamento vão lhe explicar que há três resultados possíveis para o teste:

1) Você pode ter herdado essa alteração nesses genes de predisposição ao câncer de mama e ovário. Com isso, você poderá descobrir que tem um risco aumentado de ter um segundo câncer, do mesmo tipo, ou de um tipo diferente do que já teve. Em estudos prévios, realizados em outros países, cerca de 15-35% das famílias com uma síndrome de predisposição hereditária ao câncer de mama e/ou ovário apresentavam rearranjos no gene *BRCA*;

2) Você pode não ter herdado essas alterações nesses genes de predisposição. Com isso, podemos chegar à conclusão que essas alterações não explicam sua história pessoal e familiar de câncer. Ainda assim, é possível que você tenha herdado uma *outra alteração em BRCA* ou *uma alteração em um outro gene de predisposição* para o qual ainda não há testes disponíveis;

3) Pode ser impossível de determinar se você herdou ou não um gene de predisposição alterado (teste inconclusivo). Nesta hipótese, você poderia ter herdado uma alteração em outro gene de predisposição ao câncer, para o qual não foi testado(a).

COMPLICAÇÕES E RISCOS ESPERADOS:

Normalmente há riscos mínimos envolvidos na coleta de uma amostra de sangue, que incluem dor local, sangramento, hematoma e infecção. Os riscos deste estudo são principalmente de origem psicológica para aqueles participantes que desejarem saber o resultado. Saber que você tem maior risco de desenvolver certos tipos de câncer por ter uma alteração genética poderia causar depressão, ansiedade, raiva e medo do futuro. O aconselhamento genético tem o objetivo de ajudá-lo(a) a ajustar e lidar com a informação recebida.

A Síndrome de Câncer de Mama e Ovário Hereditários (HBOC) é uma síndrome hereditária de predisposição ao câncer, que aumenta as chances de desenvolvimento de câncer de mama, ovário e outros tumores. O risco aparenta ser maior em mulheres, devido ao alto risco de desenvolvimento de câncer de mama e ovário. Esses tumores podem ser rastreados, encontrados em estágios iniciais e muitas vezes menos invasivos. O teste genético, nesse caso, visa identificar alterações (rearranjos) nos genes *BRCA1* e *BRCA2* que podem aumentar o risco para esses tumores. No entanto, em parte dos casos não é possível detectar as alterações genéticas pelos métodos atuais.

BENEFÍCIOS QUE PODERÃO SER OBTIDOS:

Este estudo permite o diagnóstico molecular de rearranjos em genes já associados ao câncer de mama e ovário. Essas alterações (rearranjos) podem levar a uma maior predisposição para o desenvolvimento desses tumores, confirmando o diagnóstico clínico. Uma vez identificada alguma alteração, o diagnóstico em familiares assintomáticos pode ser realizado, acompanhado de aconselhamento genético das famílias acometidas. A realização do teste possibilita identificar pessoas em risco e encaminhá-las para programas mais intensivos de prevenção, diminuindo desta forma os danos causados pelo tumor devido ao diagnóstico e tratamento precoce. A descoberta de alterações nos genes de predisposição e a comparação com alterações previamente descritas possibilitarão uma melhor compreensão dos mecanismos da doença e o desenvolvimento de estratégias de rastreamento mais eficazes.

HCPA / GPPG
VERSÃO APROVADA
12 / 06 / 2009

DOCUMENTAÇÃO DE CONSENTIMENTO:

1. Declaro ter sido esclarecido sobre a garantia de receber resposta a qualquer pergunta ou esclarecimento sobre procedimentos, riscos, benefícios ligados à pesquisa e ao tratamento e que serei informado quanto ao desenvolvimento de novos exames relacionados.
SIM **NÃO**

2. Declaro estar ciente de meu direito de retirar meu consentimento a qualquer momento, sem que isso traga prejuízo a continuidade de meu tratamento.
SIM **NÃO**

3. Declaro ter sido esclarecido que não receberei nenhum tipo de remuneração financeira.
SIM **NÃO**

4. Declaro ter sido esclarecido sobre a segurança de que minha identidade será preservada e que todas as informações por mim fornecidas serão confidenciais.
SIM **NÃO**

5. Autorizo o armazenamento da amostra de meu DNA, obtido neste projeto de pesquisa para utilização futura.
SIM **NÃO**

6. Declaro estar ciente de que poderei optar por não saber o resultado do teste quando este estiver disponível.
SIM **NÃO**

7. Em caso de impossibilidade de receber o resultado pessoalmente, autorizo _____ a recebê-lo.

8. Consentimento:
Eu expliquei a _____os objetivos e procedimentos necessários para este teste genético e os possíveis riscos e benefícios na minha melhor capacidade.

_____	_____	_____
Assinatura	**Nome por extenso**	**Data**

Eu li e recebi uma cópia deste formulário de consentimento. Eu concordo em realizar a análise genética e aceito os riscos. Eu entendo a informação fornecida por este documento e eu tive a oportunidade de fazer perguntas e esclarecer dúvidas que eu tinha sobre o teste, o procedimento, os riscos associados e as alternativas.

_____	_____	_____
Participante	**Data**	**Data de nascimento**

_____	_____
Testemunha	**Data**

HCPA / GPPG
VERSÃO APROVADA
12/06/2009
ive 09115

11.4 Ficha Clínica

AGC # ☐☐ ____

FICHA CLÍNICA
CÂNCER DE MAMA E/OU OVÁRIO HEREDITÁRIOS

IDENTIFICAÇÃO

1. Data: __/__/__ ☐☐☐ 2.FICHA AR.: ☐☐☐☐ 3. FAMÍLIA No.: ☐☐☐☐

4. Nome completo: _____
(em letra de forma e legível, exatamente como consta na carteira de identidade)

5. No. Prontuário: _____ ☐☐☐☐☐☐ 6. Instit.: _____ ☐

7. Nome de família materno: _____ 8. Nome de família paterno: _____

9. Endereço: _____ 10. Telefone: _____

11. Município: _____ ☐☐☐☐☐☐ 12. Estado: _____ ☐☐

13. CEP: _____ ☐☐☐☐☐ ☐☐☐ 14. Contato (tel): _____

15. Data de nascimento: __/__/__ ☐☐☐ 16. Idade: ☐☐ anos 17. Sexo: ☐ 1. Masc 2. Fem

18. Etnia: _____ ☐☐ 19. Peso: ☐☐☐ kg 20. Altura: ☐☐☐ cm

21. Naturalidade: _____ ☐☐☐☐☐☐ 22. Estado: _____ ☐☐

23. Escolaridade: ☐ 1. Analfabeto 2. Prim. incompl 24. Ocupação A. ☐☐☐ B. ☐☐☐
3. Prim. completo 4. Secund. incompl
5. Secund completo 6. Sup incompleto 7. Sup completo

25. Estado civil: ☐ 1. Solteiro(a) 2. Casado(a) 3. Viúvo(a) 4. Separado(a) 5. Outro: _____

26. Encaminhado(a) por: Dr.(a) _____ 27. Local de origem: ☐
1. Amb risco HSR 2. Amb Onco HSR 3. Amb Cir HSR 4. Outro Hospital 5. Outro: _____

DIAGNÓSTICO

28. Caso: ☐ 1. Portador de ca. de mama 2. Portador de ca. de ovário 3. Portador de ca. mama e ovário
4. Familiar com ca de mama 5. Familiar com ca de ovário 6. Familiar com ca mama e ovário

29. Médico e /ou instituição onde foi feito o diagnóstico: _____

30. Data do diagnóstico: __/__/__ ☐☐☐ 31. Localização: _____ ☐☐

32. Idade ao diagnóstico: ☐☐ anos 33. Tipo histológico: _____ ☐☐

34. Estágio: T _____ ☐ N _____ ☐ M _____ ☐

35. ACOMPANHANTES: ☐ 1. Sim 2. Não 36. Quem ? ☐ 1.Mãe 2.Pai 3. Irmão/Irmã 4.Tio/tia
5. Cônjuge 6. Amigo(a) 7. Outro

37.HDA: _____

38.HMP: _____

39.HCP: _____

RASTREAMENTO PARA CÂNCER: Realiza atualmente:

40. auto-exame da mama ☐ 1. Sim 2. Não **41. Periodicidade:** _____ ☐

42. mamografia ☐ 1. Sim 2. Não **43. Periodicidade:** _____ ☐

44. ultrassonografia TV ☐ 1. Sim 2. Não **45. Periodicidade:** _____ ☐

46. CA-125 ☐ 1. Sim 2. Não **47. Periodicidade:** _____ ☐

48. exame ginecológico ☐ 1. Sim 2. Não **49. Periodicidade:** _____ ☐

1. Semanal 2. Mensal 3. Semestral
5. Anual 6. Bi-anual 7. Outro

HISTÓRIA MASTOLÓGICA

50. Data da última mamografia: ☐☐☐ **51. Idade na 1a. mamografia:** ☐☐ anos

52. Data do último exame de mama: ☐☐☐ **53. No. total de mamografias:** ☐☐

54. Doença benigna da mama ? ☐ 1. Sim 2. Não **55. Tipo:** _____ ☐☐☐☐

56. Biópsias de mama ? ☐ 1. Sim 2. Não **57. Número de biópsias:** ☐☐

HISTÓRIA GINECOLÓGICA

58. Data do último exame pélvico: ☐☐☐ **59. Data do último preventivo:** ☐☐☐

HISTÓRIA REPRODUTIVA

60. Idade na menarca: ☐☐ anos **61. DUM:** ☐☐☐ **62. Padrão ciclo:** ☐ 1. Reg. 2. Irreg

63. Idade na menopausa: ☐☐ anos **64. Tipo:** ☐ 1. Natural 2. Gestação (amament.)
3. Induzida medic. 4. Ooforectomia bilat.
5. Induzida RxT 6. Panhisterectomia

REVISÃO DE SISTEMAS:			
65.Constitucional		72.Pele	
66.Olhos		73.Musculo-esquel.	
67.ORL		74.Neurológico	
68.Respiratório		75.Psiquiátrico	
69.Cardiovascular		76.Endocrinológico	
70.Gastrointestinal		77.Hematológico	
71.Genito-urinário		78.Imunológico	

FATORES DE RISCO PARA CÂNCER DE MAMA/OVÁRIO

79. Idade ao nascimento do 1o. filho: ☐☐ **anos** **80. G** ☐ **P** ☐ **AE** ☐ **AI** ☐

HISTÓRIA GESTACIONAL

GESTA	Ano/mês Fim gesta	Resultado 1. NV 2. NM 3. ABOE 4. ABOI	Gesta mult 1. Sim 2. Não	Amament. 1. Sim 2. Não	Duração (m) amament.
1	81.	82.	83.	84.	85.
2	86.	87.	88.	89.	90.
3	91.	92.	93.	94.	95.
4	96.	97.	98.	99.	100.
5	101.	102.	103.	104.	105.

106. USO DE ESTRÓGENOS EXÓGENOS ☐ 1. Sim 2. Não

107. Tipo ☐ 1. ACO 2. TRH 3. Outro

108. Nome comercial: ☐☐

109. Duração do uso: ☐☐ meses

110. Forma ☐ 1. VO 2. EV 3. IM

111. Tipo: ☐ 1. ACO 2. TRH 3. Outro

112.Nome comercial: ☐☐

113. Duração do uso: ☐☐ meses

114. Forma ☐ 1. VO 2. EV 3. IM

115. CONSUMO DE ÁLCOOL ☐ 1. Sim 2. Não

116. Tipo: ☐ 1. Cerveja 2. Vinho 3. Destilados

117. Volume: ☐☐ copos/doses

118. Freqüência: ☐ 1. Diário 2. Semanal 3. Mensal 4. Anual

119. Tipo: ☐ 1. Cerveja 2. Vinho 3. Destilados

120. Volume: ☐☐ copos/doses

121. Freqüência: ☐ 1. Diário 2. Semanal 3. Mensal 4. Anual

122. FUMO ☐ 1. Sim 2. Não **123. Há** ☐☐ anos **124. No. cigarros/dia** ☐☐

125. EXPOSIÇÃO À RADIAÇÃO: ☐ 1. Sim 2. Não

126. Número de raios X: ☐☐ **antes dos 20 anos** **127.** ☐☐ **após os 20 anos**

128. Outra exposição a radiação ionizante: ☐

AGC # ☐☐☐☐

EXAME FÍSICO: _____

AVALIAÇÃO RADIOLÓGICA: _____

CONSULTORIAS: _____

IMPRESSÃO: _____

165. RECOMENDAÇÃO QTO. TESTE GENÉTICO: ☐ 1. INDICADO 2. NÃO INDICADO

PLANO: _____

RECOMENDAÇÕES DE PREVENÇÃO/DIAGNÓSTICO PRECOCE: _____

ASPECTOS ESPECIAIS DO ACONSELHAMENTO GENÉTICO: _____

166. DIAGNÓSTICO: _____

ATENDIDO POR: _____ CREMERS: _____

AGC # ___ ___ ___

CONSULTAS DE SEGUIMENTO

CONSULTA # 2 **DATA: ___/___/___ ACOMPANHANTES:**_____

CONSULTA # 3 **DATA: ___/___/___ ACOMPANHANTES:**_____

CONSULTA # 4 **DATA: ___/___/___ ACOMPANHANTES:**_____

CONSULTA # 5 **DATA: ___/___/___ ACOMPANHANTES:**_____

11.5 Protocolos Laboratoriais

11.5.1 Caracterização dos pontos de quebra dos rearranjos identificados, realizados no Instituto de Oncologia do Porto – IPO, Porto, Portugal

***Mix* da reação de PCR** *(BRCA* 1 exons9-19)

Reagente	Concentração de uso	Volume final por amostra (ul)	Concentração final
H_2O	-	19,9	-
Tampão	10X	3	1X
$MgCl_2$	50 mM	3	3 mM
dNTP	1uM	0,3	0.02 uM
Primer F	20 uM	1,5	0.4 uM
Primer R	20 uM	1,5	0.4 uM
Taq platinum	5 U/ul	0.2	0.01 U/ul
DNA	100 ug/ul	1,0	-
	Volume final:	30ul	

***Mix* da reação de PCR** *(BRCA* 1 exons16-17)

Reagente	Concentração de uso	Volume final por amostra (ul)	Concentração final
H_2O	-	15,5	-
Tampão	10X	1,5	1X
$MgCl_2$	50 mM	1,0	3 mM
dNTP	1uM	1,0	0.02 uM
Primer F	20 uM	0,3	0.4 uM
Primer R	20 uM	0,3	0.4 uM
Taq gold – applied Bios.	5 U/ul	0,2	0.01 U/ul
DNA	100 ug/ul	0,2	-
	Volume final:	20ul	

Programa de termociclador: Applied Biosystens (Del 9-19)

Temperatura (°C)	Tempo	Número de ciclos
97	15min	1
97	1min	
68	1min	6
72	1min	
97	1min	
66	30seg	6
72	1min	
97	1min	
64	30seg	6
72	1min	

Programa de termociclador: Applied Biosystens (Del 16-17)

Temperatura (°C)	Tempo	Número de ciclos
95	5min	5
94	1min	
56	1min	5
72	1min	
94	1min	
54	1seg	5
72	1min	
94	1min	
50	1min	25
72	1min	
72	10min	1

Programa de termociclador: Mastercycler – Eppendorf (Del exon 19)

Temperatura (°C)	Tempo (minutos)	Número de ciclos
94	5min	1
94	3min	
60	1min	34
72	1min	
72	10min	1
10	20min	1

Primers utilizados: **Metabion**– 20 pmol,

BRCA1 –exon 19

5'-TCTATCTCCGTGAAAAGAG 3' and 5'-CTGGTTAGTTTGTAACATC -3'

BRCA1 – exons 9-19

5'-ACTCTGAGGACAAAGCAGCGGA-3'and 5'- GTGCCACCAAGCCCGGCTAA -3'

BRCA1 – exons 16-17

5'-TGAGGATGAGGGAGTCTTGGTGTAC -3 and
5'TCCTAAACACAGCAGGCTATCTGCA-3'-

Protocolo de sequenciamento gênico:

Após verificar os produtos de PCR *(feito em gel de agarose 2%e Ladder – Invitrogen)* para checar os produtos em tamanho (bp) de cada fragmento a ser amplificado.

Efetuou-se a purificação dos mesmos através do *kit: Ilustras GE Helthcare UK Limited, Buckinghamshire, United Kingdom).* Os produtos de PCR ou as bandas retiradas do gel foram purificados segundo o protocolo recomendado pelo fabricante. Para avaliar a qualidade e quantidade dos produtos purificados, submeteram-se os produtos a electroforese em gel de agarose a 2% (p/v) corado com brometo de etídeo.

Reação de sequenciamento:

Pré mix	1 ul
Primer (5μM)	0,35μL
buffer	1,9μL
Amostra	4μL
H_2O Milli-Q	3,75μL
Volume total	10μL (pode exceder)

Programa sequenciamento termociclador:

Ciclar:	95°C	20"	30 ciclos
	55°C	15"	
	60°C	1'30"	

Os produtos de seqüenciamento foram precipitados e purificados com acetato de sódio e etanol (Merck) de acordo com protocolos convencionais. Em seguida as amostras foram analisadas em sequenciador automático *ABI PRISM TM 310 Genetic Analyser*.

11.5.2 Protocolo de amplificação para detecção de mutação fundadora portuguesa c.156_157insAlu – *BRCA2*

***Mix* da reação de PCR** *(BRCA*2 exon 3)

Reagente	Concentração de uso	Volume final por amostra (ul)	Concentração final
H_2O	-	19,9	-
Tampão	10X	3	1X
$MgCl_2$	50 mM	3	3 mM
dNTP	1uM	0,3	0.02 uM
Primer F	20 uM	1,5	0.4 uM
Primer R	20 uM	1,5	0.4 uM
Taq platinum	5 U/ul	0.2	0.01 U/ul
DNA	100 ug/ul	1,0	-
	Volume final:	30ul	

Programa de termociclador: Mastercycler – Eppendorf

Temperatura (°C)	Tempo	Número de ciclos
97	15min	1
97	1min	
68	1min	6
72	1min	
97	1min	
66	30seg	6
72	1min	
97	1min	
64	30seg	6
72	1min	

Primers utilizados: **Invitrogen** – 20 pmol,

1° PCR exon 3 do gene BRCA2 :

5'gtc act ggt taa aac taa ggt ggg a 3' and 5' gaa gcc agc tga tta taa gat ggt t 3'

2° PCR específico c. 156_157insAlu *BRCA2:*
(ALU): 5'gac acc atc ccg gct gaa a3' and ccc cag tct acc ata ttg cat 3'

Programa de termociclador: PCR Mastercycler – Eppendorf

	94ºC	00:01:00	
94ºC 0:03:00	52ºC	00:01:00	72ºC 0:10:00
	72ºC	00:04:00	
	35 cycles		

11.5.3 Protocolo de amplificação de MLPA (MRC-Holland)

1° Etapa: Hibridização
Em tubos de 0,2ml - as amostras de DNA: 5ul por amostra (com concentração média de 50ng/ul com H2O)

*Desnaturar as amostras a 98°C por 5 minutos no termociclador.

*Adicionar à amostra 1,5ul de SALSA probemix (black cap) + 1.5 3l MLPA buffer (yellow cap) para cada tudo.

*Incubar por 1 minuto a 95 °C, durante 16 hrs a 60 °C.

2° Etapa: Reação de Ligação
*Reduzir a tempreratura do termo a 54°C: adicionar 3ul de buffer A + 3ul de buffer B + 25 ul de H2O + 1ul de ligase 65 (colocar 32ul desse mix por amostra)

*Incubar 15 minutos a 54 °C, e 5 minutes at 98 °C.

3° Etapa: Reação de PCR
*4 ul de SALSA PCR buffer + 26 ul de H2O + 10ul da reação de ligação

[Colocar esse mix (30ul no total por amostra) em novos tubos de 0,2ml

*Posteriormente colocar 10ul do produto de ligação que está no termo e aquecer a 60°C durante 5 minutos.

Por fim:

*2ul (salsa PCR primers)
*2ul (SALSA Enzyme Dilution buffer)
*5,5ul de H2O
*0,5ul (SALSA Polymerase)
Adicionar 10ul do mix para cada amostra e deixar no termo com o seguinte programa de PCR:

35 ciclos: 30 segundos 95°C; 30 segundos 60°C; 60 segundos 72°C e 20 minutes incubando a 72°C.

11.5.4 Mix de sondas kit P002B (MRC-Holland) para análise de rearranjos por MLPA no gene *BRCA1*

Table 1. SALSA MLPA P002 BRCA1 probemix

Length (nt)	SALSA MLPA probe	Chromosomal position reference	Chromosomal position BRCA1
64-70-76-82	Q-fragments: DNA quantity; only visible with less than 100 ng sample DNA		
88-92-96	D-fragments: Low signal of 88 or 96 nt fragment indicates incomplete denaturation		
100	X-fragment: Specific for the X chromosome		
105	Y-fragment: Specific for the Y chromosome		
127	Reference probe 00797-L00093	5q31	
136	Reference probe 06452-L05978	6p22	
148	**BRCA1 probe** 00763-L00268		**exon 1a**
157	**BRCA1 probe** 00764-L00269		**exon 1b**
166	**BRCA1 probe** 00765-L00270		**exon 2**
175	**BRCA1 probe** 00826-L00341		**exon 3**
184	**BRCA1 probe** 00767-L00272		**exon 5**
198	Reference probe 02946-L03265	7q31	
208	**BRCA1 probe** 00827-L00342		**exon 6**
216	**BRCA1 probe** 00769-L00274		**exon 7**
226	**BRCA1 probe** 01004-L00569		**exon 8**
236	**BRCA1 probe** 01005-L00581		**exon 9**
244	**BRCA1 probe** 00772-L00277		**exon 10**
256	Reference probe 00518-L00098	2q13	
266	**BRCA1 probe** 00830-L00345		**exon 11**
277	**BRCA1 probe** 00774-L00279		**exon 11**
285	**BRCA1 probe** 00775-L00280		**exon 12**
295 ±	**BRCA1 probe** 02603-L02074		**exon 13**
305	**BRCA1 probe** 00833-L00349		**exon 14**
316	Reference probe 00495-L00303	12p12	
328	**BRCA1 probe** 00778-L00347		**exon 15**
337	**BRCA1 probe** 00779-L00003		**exon 16**
346	**BRCA1 probe** 00780-L00283		**exon 17**
355 ±	**BRCA1 probe** 00781-L00284		**exon 18**
364	**BRCA1 probe** 00782-L00285		**exon 19**
374	Reference probe 00655-L00304	4q27	
389	**BRCA1 probe** 00783-L00356		**exon 20**
399	**BRCA1 probe** 00784-L12004		**exon 21**
407	**BRCA1 probe** 00785-L00288		**exon 22**
415	**BRCA1 probe** 00786-L00289		**exon 23**
427 ±	**BRCA1 probe** 02831-L13862		**exon 24**
436	Reference probe 00596-L00083	11p13	
445	Reference probe 04074-L03710	17q11	
454	Reference probe 00673-L00117	3p22	
463 ±	**BRCA1 probe** 11283-L12001		**exon 13**

± Probe has a higher standard variation.

Note: The exon numbering in Table 1 is different as compared to the exon numbering used by the NCBI in the NM_007294.3 reference sequence! We used the same exon numbering as in all previous versions of this product description. Please notify us of any mistakes. The identity of the genes detected by the reference probes is available on request: info@mlpa.com.

11.5.5 Mix de sondas kit P087 (MRC-Holland) para análise de rearranjos por MLPA no gene *BRCA1*

Table 1. SALSA MLPA P087-B1 BRCA1 probemix

Length (nt)	SALSA MLPA probe	Chromosomal position reference	BRCA1
64-70-76-82	Q-fragments: DNA quantity; only visible with less than 100 ng sample DNA		
88-92-96	D-fragments: Low signal of 88 or 96 nt fragment indicates incomplete denaturation		
100	X-fragment: Specific for the X chromosome		
105	Y-fragment: Specific for the Y chromosome		
130	Reference probe 02269-L01761	1p36	
136	Reference probe 02283-L01774	13q13 *(BRCA2, exon 1)*	
148	**BRCA1 probe** 02807-L01268		**Promoter region**
157	**BRCA1 probe** 02808-L02168		**Promoter region**
167	**BRCA1 probe** 02810-L02239		**Exon 2**
175	**BRCA1 probe** 02811-L02240		**Exon 3**
185	**BRCA1 probe** 03398-L02254		**Exon 18**
193	Reference probe 03217-L02642	10q25	
200 *	**BRCA1 probe** 11457-L12189		**Exon 22**
208	**BRCA1 probe** 02813-L02242		**Exon 6**
219	**BRCA1 probe** 02814-L02243		**Exon 7**
226	**BRCA1 probe** 02815-L02244		**Exon 8**
234	**BRCA1 probe** 02816-L02245		**Exon 9**
244	**BRCA1 probe** 03411-L02074		**Exon 13**
256	Reference probe 02279-L01770	13q13 *(BRCA2, exon 11)*	
263 *	**BRCA1 probe** 11802-L12190		**Exon 14**
276	**BRCA1 probe** 02818-L02247		**Exon 11**
287	**BRCA1 probe** 02819-L02248		**Exon 12**
295	**BRCA1 probe** 03890-L03337		**Exon 13**
310 *	Reference probe 09809-L10644	13q13 *(BRCA2, exon 5)*	
319	Reference probe 00495-L03128	12p12	
329	**BRCA1 probe** 02821-L02250		**Exon 15**
337	**BRCA1 probe** 02822-L02251		**Exon 16**
346 ¥	**BRCA1 probe** 03395-L12877		**Exon 5**
355	**BRCA1 probe** 03822-L03285		**Exon 10**
364	**BRCA1 probe** 02826-L02255		**Exon 19**
371	Reference probe 02667-L02134	11q22 *(ATM, exon 25)*	
380	Reference probe 00655-L03268	4q27	
390	**BRCA1 probe** 02827-L02256		**Exon 20**
397	**BRCA1 probe** 02828-L02257		**Exon 21**
408 ¥	**BRCA1 probe** 03397-L13116		**Exon 17**
416	**BRCA1 probe** 02830-L02259		**Exon 23**
425 *	**BRCA1 probe** 04578-L04795		**Exon 24**
436 ¥	**BRCA1 probe** 02100-L02537		**Exon 1**
445	Reference probe 02445-L01409	16p13	
454	Reference probe 02355-L01415	9q34	

* New in version B1 (lot 0508 onwards)
¥ Changed in version B1. Small change in length or peak height. No change in sequence detected.

Note: The exon numbering in Table 2 is different as compared to the exon numbering used by the NCBI in the NM_007294.3 reference sequence! We used the same exon numbering as in all previous versions of this product description. Please notify us of any mistakes. The identity of the genes detected by the reference probes is available on request: info@mlpa.com.

Please note that two probes for exon 13 are present. The 244 nt probe (03411-L02074) detects the same sequence as the exon 13 probe at 295 nt in SALSA MLPA P002 probemix.

11.5.6 Mix de sondas kit P045 (MRC-Holland) para análise de rearranjos por MLPA no gene *BRCA2*

Table 1. SALSA MLPA P045-B3 BRCA2/CHEK2 probemix

Length (nt)	SALSA MLPA probe	Chromosomal position		
		reference	CHEK2	BRCA2
64-70-76-82	Q-fragments: DNA quantity; only visible with less than 100 ng sample DNA			
88-92-96	D-fragments: Low signal of 88 or 96 nt fragment indicates incomplete denaturation			
100	X-fragment: Specific for the X chromosome			
105	Y-fragment: Specific for the Y chromosome			
130	Reference probe 00797-L00463	5q31		
137	**BRCA2 probe** 02283-L12281			**Exon 1**
148	**BRCA2 probe** 02285-L01776			**Exon 1**
154	**BRCA2 probe** 09297-L08066			**Exon 14**
160	**FRY probe** 02143-L09586			20 kb before BRCA2
166	**BRCA2 probe** 02486-L01985			**Exon 2**
172 ∞	**BRCA2 probe** 08898-L09587			**Exon 3**
178	**BRCA2 probe** 01599-L10642			**Exon 3**
184	Reference probe 01217-L00694	4q35		
191	**BRCA2 probe** 09812-L10643			**Exon 23**
197	**BRCA2 probe** 01600-L04671			**Exon 4**
202	**BRCA2 probe** 08265-L08128			**Exon 7**
211	Reference probe 02333-L01826	12q23		
220	**BRCA2 probe** 01602-L01184			**Exon 8**
229	**BRCA2 probe** 01603-L01185			**Exon 9**
238	Reference probe 00517-L00097	2q13		
247	**BRCA2 probe** 01604-L01186			**Exon 10**
256 °	**BRCA2 probe** 02279-L01770			**Exon 11** start
265	**HCS20 probe** 06800-L02040		In CHEK2 promotor region	
274	**BRCA2 probe** 01606-L01188			**Exon 11** end
283	**BRCA2 probe** 01607-L01189			**Exon 12**
292	Reference probe 03018-L02458	12q13		
301	**BRCA2 probe** 02280-L01771			**Exon 13**
310	**BRCA2 probe** 09809-L10257			**Exon 5**
319 ±	**BRCA2 probe** 09296-L11090			**Exon 27**
326	**BRCA2 probe** 01610-L01192			**Exon 15**
337	**BRCA2 probe** 01611-L01193			**Exon 16**
346	**BRCA2 probe** 04585-L03983			**Exon 6**
355	**BRCA2 probe** 02281-L01772			**Exon 17**
364 ~	**BRCA2 probe** 01613-L01195			**Exon 18**
373	Reference probe 02667-L04984	11q22		
382	**BRCA2 probe** 01614-L01196			**Exon 19**
391	**BRCA2 probe** 08266-L08129			**Exon 20**
400 ^‡	**CHEK2 probe** 02579-L12282		**CHEK2 Exon 10**	
409	**BRCA2 probe** 02069-L01970			**Exon 21**
418	**BRCA2 probe** 01617-L01199			**Exon 22**
427	Reference probe 06942-L06522	11q12		
436	**BRCA2 probe** 08267-L08130			**Exon 24**
445	**BRCA2 probe** 08268-L08131			**Exon 25**
454	**N4BP2L1 (CG018) probe** 02144-L01619			9 kb after BRCA2
463	**BRCA2 probe** 11984-L15346			**Exon 26**
476 ±	**BRCA2 probe** 09293-L15678			**Exon 27**
486	Reference probe 05028-L15679	2q32		
495 §^	**CHEK2 probe** 01772-L15680		**CHEK2 exon 12, Mutation 1100delC specific!**	

^ The CHEK2 exon numbering has changed. We now use the exon numbering as present in the NCBI NM_001005735 reference sequence.

‡ This probe is located within, or close to, a very strong CpG island. A low signal of this probe can be due to incomplete sample DNA denaturation, e.g. due to the presence of salt in the sample DNA.

§ Mutation 1100delC-specific! This peak will only appear if the point mutation is present.

Legend continues below Table 2.