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Spectrum of *BRCA1* and *BRCA2* germline mutations in Portuguese Hereditary Breast and Ovarian cancer: ancestral origin of founder mutations and their implications for genetic testing criteria and strategy

Dissertation for applying to a Doctoral degree in Biomedical Sciences submitted to the Institute of Biomedical Sciences Abel Salazar of the University of Porto.

Host Institution - Department of Genetics, Portuguese Oncology Institute of Porto.

Aos meus Pais

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TABLE OF CONTENTS

SUMMARY

Inherited predisposition to breast cancer is estimated to account for 5–10% of all cases and is characterized by an autosomal dominant pattern of inheritance, young age at presentation, and association with bilateral and male breast cancer and ovarian cancer. Germline pathogenic mutations in the *BRCA1* and *BRCA2* genes are responsible for the Hereditary Breast and Ovarian Cancer Syndrome (HBOC). The pattern of *BRCA1* and *BRCA2* mutations in 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 and/or recurrent effects. This is the case of the c.156_157insAlu *BRCA2* rearrangement, which was first identified in a Portuguese patient residing in Belgium and later described as a founder mutation in HBOC families mostly originated from central/southern Portugal.

This study aimed to characterize the mutational spectrum and to evaluate the impact of founder mutations in the genetic testing criteria and strategy for molecular testing of HBOC families of Portuguese ancestry. In an attempt to gain insight into the ancestral origin and population spread of the c.156_157insAlu *BRCA2* mutation, we estimated its frequency in breast/ovarian cancer families originated mostly from northern/central Portugal. 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 5,294 suspected HBOC families living in countries other than Portugal in whom no deleterious *BRCA1*/*BRCA2* mutations had previously been found. Furthermore, we characterized the pathological and clinical significance of the c.156_157insAlu *BRCA2* mutation. Haplotype analysis using microsatellite markers was performed for the *BRCA2* c.156_157insAlu mutation, as well for two other *BRCA1* mutations (c.3331_3334del and c.2037delinsCC). Breakpoint identification of Large Genomic Rearrangements (LRGs) detected in Portuguese HBOC families was also part of the scope of this work.

Our results indicate that inherited cancer predisposition can be linked to *BRCA1* or *BRCA2* mutations in about 30% of the Portuguese breast/ovarian cancer families. Despite the mutational spectrum heterogeneity of germline *BRCA1*/*BRCA2* mutations in Portuguese breast/ovarian cancer families, three variants (c.156_157insAlu in the *BRCA2* gene and c.3331_3334del and

c.2037delinsCC in the *BRCA1* gene) together account for about 50% of all pathogenic mutations. The *BRCA2* c.156_157insAlu rearrangement is a Portuguese founder mutation originated about 558 ± 215 years ago and has been reported only in HBOC families from Portugal or of Portuguese ancestry. Families with the *BRCA1* c.3331_3334del or the c.2037delinsCC mutations each share a common haplotype, indicating that these are also founder mutations in the Portuguese population. Concerning the pathological and clinical significance of the c.156_157insAlu mutation, we concluded that this mutation is deleterious for the following reasons: it originates *BRCA2* exon 3 skipping; the *BRCA2* full length transcript is derived only from the wild type allele in carriers; it is absent in disease free controls; it co-segregates with the disease; and the cumulative incidence of breast cancer in mutation carriers does not differ from that of other *BRCA2* and *BRCA1* pathogenic mutations. In addition to point mutations, two novel *BRCA1* LRGs were detected in Portuguese HBOC families and we were able to identify its breakpoints, one encompassing exons 1–7 in addition to the two adjacent genes *NBR2* and *NBR1* (c.441+1724_oNBR1:c.1073+480del) and another comprising exons 11–15 (c.671-319_4677-578delinsAlu).

Based on the findings of this thesis, we recommend offering genetic testing of the two most common mutations (c.156_157insAlu in the *BRCA2* gene and c.3331_3334del in the *BRCA1* gene) to any affected proband with the following clinical criteria: any breast cancer patient diagnosed until the age of 35 (or 40 in case of a triple negative or medullary carcinoma); two first/second degree relatives with breast or ovarian cancer, at least one diagnosed before the age of 50 (or pancreatic cancer at any age); three first/second degree relatives with breast, ovarian or pancreatic cancer at any age; any male breast cancer. If no founder mutations are found, screening of the entire coding regions of *BRCA1* and *BRCA2* should be offered to those families with an *a priori* mutation probability ≥10%.

RESUMO

A predisposição hereditária para cancro da mama representa cerca de 5-10% da totalidade dos casos de cancro da mama e caracteriza-se por um padrão de transmissão autossómico dominante, idade precoce de diagnóstico e associação a cancro da mama bilateral, cancro do ovário e cancro da mama no homem. Mutações germinativas patogénicas nos genes *BRCA1* e *BRCA2* predispõem para a síndrome de cancro da mama/ovário hereditário (Hereditary Breast and Ovarian Cancer – HBOC). O padrão de mutações na síndrome HBOC é muito heterogéneo, no entanto determinadas populações ou grupos étnicos apresentam mutações específicas com elevada frequência devido a efeitos fundadores e/ou recorrentes. É o caso da mutação c.156_157insAlu no gene *BRCA2* inicialmente identificada num indivíduo de origem portuguesa a residir na Bélgica e posteriormente descrita como mutação fundadora em famílias HBOC portuguesas originárias da região centro/sul de Portugal.

Este estudo teve como objetivos principais caracterizar o espectro mutacional dos genes *BRCA1* e *BRCA2* e avaliar o impacto das mutações fundadoras nos critérios utilizados na seleção de famílias para a realização de estudo genético, assim como no diagnóstico molecular da síndrome HBOC em famílias com ancestralidade portuguesa. Desta forma, fomos estudar a frequência da mutação c.156_157insAlu em famílias com critérios HBOC, originárias, na sua maioria, da região norte/centro de Portugal, e avaliar se essas famílias partilhavam um ancestral comum. Dado que esta mutação não é detetável com as metodologias normalmente utilizadas no diagnóstico molecular desta síndrome, fomos pesquisar este rearranjo em 5,294 famílias com critérios HBOC provenientes de outros países e nas quais não se tinham identificado mutações patogénicas nos genes *BRCA1*/*BRCA2*. Foi também realizada a avaliação da patogenicidade e do significado clínico desta mutação. A análise de haplótipos, utilizando marcadores microssatélite, foi efetuada para a mutação c.156_157insAlu no gene *BRCA2*, assim como para as mutações c.3331_3334del e c.2037delinsCC no gene *BRCA1*. A caracterização dos pontos de quebra dos rearranjos genómicos detetados na série de famílias HBOC analisadas também fez parte do âmbito deste trabalho.

Os nossos resultados indicam que a predisposição hereditária para cancro da mama/ovário está associada aos genes *BRCA1*/2 em 30% das

famílias portuguesas com critérios HBOC. Apesar da elevada heterogeneidade mutacional encontrada, 50% das mutações patogénicas identificadas nestas famílias são devidas a apenas três mutações: c.156_157insAlu no gene *BRCA2,* c.3331_3334del e c.2037delinsCC no gene *BRCA1*. O rearranjo c.156_157insAlu no gene *BRCA2* é uma mutação fundadora, apenas presente em famílias com ancestralidade portuguesa, que ocorreu há cerca de 558 ± 215 anos. As famílias com as mutações c.3331_3334del ou c.2037delinsCC no gene *BRCA1* também partilham, cada uma delas, um haplótipo comum, o que indica que estas alterações também são fundadoras na população portuguesa. No que diz respeito à patogenicidade e significado clínico da mutação c.156_157insAlu, concluímos que esta alteração é patogénica pelas seguintes razões: origina a remoção do exão 3 do transcrito; o transcrito que engloba a sequência íntegra do gene *BRCA2* deriva apenas do alelo normal nos portadores; está ausente em controlos normais; segrega com a doença; e a incidência cumulativa de cancro da mama em portadores não difere da descrita para outras mutações patogénicas nos genes *BRCA1*/*2*. Finalmente, foram identificados os pontos de quebra de dois rearranjos detetados em famílias portuguesas HBOC, um deles envolvendo os exões 1 a 7 do gene *BRCA1* e os genes adjacentes *NBR2* e *NBR1* (c.441+1724_oNBR1:c.1073+480del), e outro que abrange os exões 11 a 15 do gene *BRCA1* (c.671-319_4677-578delinsAlu).

Baseado nos resultados desta tese, recomendamos a pesquisa das duas mutações mais frequentes (c.156_157insAlu no gene *BRCA2* e c.3331_3334del no gene *BRCA1*), em qualquer probando afetado que preencha os seguintes critérios: doente com cancro da mama diagnosticado até à idade de 35 anos (ou 40 no caso de ser um triplo negativo ou carcinoma medular); dois familiares em primeiro/segundo grau com cancro da mama ou ovário, um deles diagnosticado antes dos 50 (ou cancro do pâncreas em qualquer idade); três familiares em primeiro/segundo grau com cancro da mama, ovário ou pâncreas em qualquer idade; cancro da mama no homem. O screening completo das regiões codificantes dos genes *BRCA1*/*2* deverá ser efetuado nos probandos em que não se encontraram mutações fundadoras e que apresentam uma probabilidade *a priori* de mutação ≥10%.

LIST OF PUBLICATIONS

According to "Artigo nº 34, do Decreto-Lei nº 115/2013", results contained in the following published works were used for this thesis:

1. Ana Peixoto, Catarina Santos, Patrícia Rocha, Manuela Pinheiro, Sofia Príncipe, Deolinda Pereira, Helena Rodrigues, Fernando Castro, Joaquim Abreu, Leonor Gusmão, António Amorim, Manuel R. Teixeira. **"The c.156_157insAlu *BRCA2* rearrangement accounts for more than one-fourth of deleterious BRCA mutations in northern/central Portugal"**. Breast Cancer Res Treat 2009, 114:31-38.

2. Ana Peixoto, Catarina Santos, Patrícia Rocha, Pedro Pinto, Susana Bizarro, Manuel R. Teixeira. **"Molecular diagnosis of the Portuguese founder mutation *BRCA2* c.156_157insAlu".** Breast Cancer Res Treat 2009, 117:215-217.

3. Ana Peixoto, Catarina Santos, Manuela Pinheiro, Pedro Pinto, Maria José Soares, Patrícia Rocha, Leonor Gusmão, António Amorim, Annemarie van der Hout, Anne-Marie Gerdes, Mads Thomassen, Torben A. Kruse, Dorthe Cruger, Lone Sunde, Yves-Jean Bignon, Nancy Uhrhammer, Lucie Cornil, Etienne Rouleau, Rosette Lidereau, Drakoulis Yannoukakos, Maroulio Pertesi, Steven Narod, Robert Royer, Maurício M. Costa, Conxi Lazaro, Lidia Feliubadaló, Begoña Graña, Ignacio Blanco, Miguel de la Hoya, Trinidad Caldés, Philippe Maillet, Gaelle Benais-Pont, Bruno Pardo, Yael Laitman, Eitan Friedman, Eladio A. Velasco, Mercedes Durán, Maria-Dolores Miramar, Ana Rodriguez Valle, María-Teresa Calvo, Ana Vega, Ana Blanco, Orland Diez, Sara Gutiérrez-Enríquez, Judith Balmaña, Teresa Ramony Cajal, Carmen Alonso, Montserrat Baiget, William Foulkes, Marc Tischkowitz, Rachel Kyle, Nelly Sabbaghian, PatriciaAshton-Prolla, Ingrid P. Ewald, Thangarajan Rajkumar, Luisa Mota-Vieira, Giuseppe Giannini, Alberto Gulino, Maria I. Achatz, Dirce M. Carraro, Brigitte Bressac de Paillerets, Audrey Remenieras, Cindy

Benson, Silvia Casadei, Mary-Claire King, Erik Teugels, Manuel R. Teixeira. **"International distribution and age estimation of the Portuguese *BRCA2* c.156_157insAlu founder mutation"**. Breast Cancer Res Treat 2011, 127:671-679.

4. Ana Peixoto, Manuela Pinheiro, Lígia Massena, Catarina Santos, Pedro Pinto, Patrícia Rocha, Carla Pinto, Manuel R Teixeira. **"Genomic characterization of two large Alu-mediated rearrangements of the *BRCA1* gene".** Journal of Human Genetics 2013, 58:78-83.

5. Ana Peixoto, Catarina Santos, Pedro Pinto, Manuela Pinheiro, Patrícia Rocha, Carla Pinto, Susana Bizarro, Isabel Veiga, Sofia A. Príncipe, Sofia Maia, Fernando Castro, Rosário Couto, Alfredo Gouveia, Manuel R. Teixeira. **"The role of targeted *BRCA1*/*BRCA2* mutation analysis in hereditary breast/ovarian cancer families of Portuguese ancestry".** Clin Genet 2015, 88:41-8.

LIST OF ABBREVIATIONS

ASR	Age-standardized rate
BASC	BRCA1-associated genome-surveillance complex
BER	Base excision repair pathway
BIC	Breast Cancer Information Core
BRCT	BRCA1 C-terminal
DBD	DNA binding domain
DCIS	Ductal carcinoma *in situ*
DSBs	Double-strand breaks
ER	Estrogen receptor
HBOC	Hereditary breast and ovarian cancer syndrome
HD	Helical domain
HER2	Human epidermal growth factor receptor 2
HR	Homologous recombination
LGR	Large gene rearrangement
LOH	Loss of heterozygosity
MLPA	Multiplex ligation probe amplification
MRI	Magnetic resonance imaging
MRN	MRE11-RAD50-NBS1 complex
NCCN	National comprehensive cancer network
NICE	National Institute for Health and Care Excellence
NHEJ	Nonhomologous end joining
NST	No special type
OB	Oligonucleotide binding
PBSO	Prophylactic bilateral salpingo-oophorectomy
PCR	Polymerase chain reaction
PM	Prophylactic mastectomy
PR	Progesterone receptor
RING	Really interesting new gene
RPA	Replication protein A
SEER	Surveillance, epidemiology, and end results program
SERMs	Selective estrogen receptor modulators
SNP	Single nucleotide polymorphism

LIST OF ABBREVIATIONS

ssDNA	Single-strand DNA
TD	Tower domain
TN	Triple-negative
TSG	Tumor suppressor genes
VUS	Variants of uncertain significance

INTRODUCTION

1. Epidemiology of breast and ovarian cancer

Breast cancer is the most frequent cancer among women, with an estimated incidence of 1.67 million new cancer cases diagnosed in 2012 worldwide (25% of all cancers), and ranks second overall (11.9% of all cancers in both sexes). Although breast cancer may occur in men, it is rare. Incidence rates are high in developed regions of the world (96 per 100,000 in Western Europe) and lower (less than 40 per 100,000) in most of the developing regions. Breast cancer is the leading cause of death by cancer in women in less developed regions and the second cause of cancer death in more developed regions after lung cancer. In Portugal, breast cancer is the most frequent cancer among women, with an estimated number of 6,088 women diagnosed in 2012, followed by colorectal cancer. In terms of cancer mortality, breast cancer is the main cause of death by cancer in Portuguese women, being estimated that 1,570 female breast cancer deaths occur annually (Figure 1) (Ferlay et al., 2013).

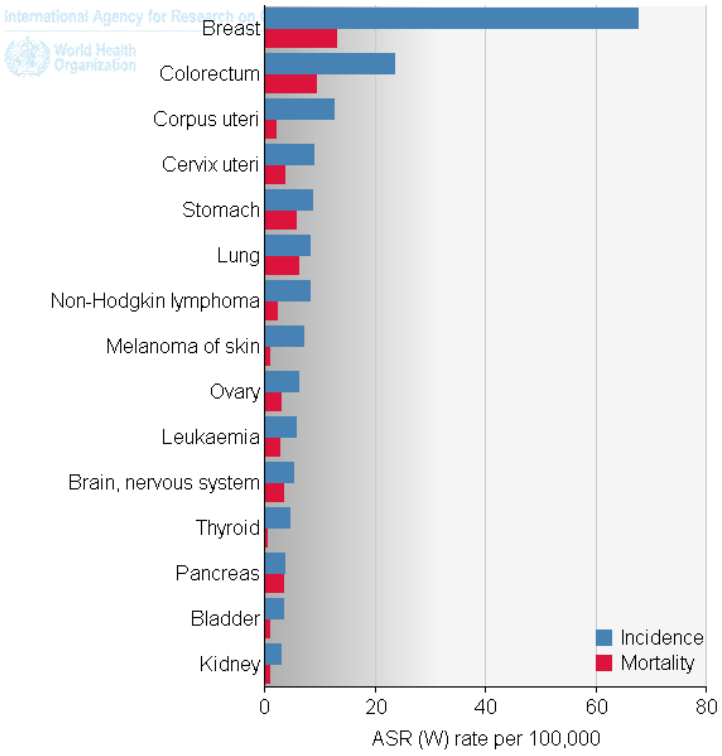

Figure 1 – Incidence and mortality age standardized rates (ASR) in women in Portugal in 2012 (Ferlay et al., 2013).

Ovarian cancer is the seventh most common cancer worldwide among women, with 238,719 new cancer cases diagnosed in 2012 (3.6% of all cancers) (Ferlay et al., 2013) (Figure 1). In Portugal, ovarian cancer represents approximately 3% of all cancers diagnosed in women, being estimated that 616 new cancer cases were diagnosed in 2012, with an estimated age-standardized rate (ASR) incidence of 6.2 per 100,000. Ovarian cancer is the sixth cause of cancer death in Portuguese females (Figure 1) (Ferlay et al., 2013).

Female breast cancer and ovarian cancer are most common in middle-aged and older women with a median age at diagnosis of 61 and 63 years old, respectively (SEER Stat Fact Sheets, 2008-2012a; SEER Stat Fact Sheets, 2008-2012b).

2. Breast and ovarian cancer risk factors

Non-modifiable breast cancer risk factors include gender, age, personal cancer history, family cancer history and genetics, race, ethnicity, early menarche, late menopause, breast density and breast benign lesions (Hulka and Moorman, 2001). Gender is the greatest risk factor for breast cancer, being at least 100 times more frequent in women than in men. The risk of breast cancer increases throughout a woman`s lifetime, with incidence rates of breast cancer increasing rapidly with age during the reproductive years (until ages 45 to 50), and then increases at a slower rate probably reflecting the impact of hormonal change (menopause) (Key and Pike, 1988). Women with a history of breast cancer are at increased risk for developing a second breast cancer. Familial aggregation is present in about 20% of the cases and can be attributed to genetic, environmental, and lifestyle factors. Given the high incidence of breast cancer in the general population, it is not uncommon for a woman to have a first degree relative with a history of breast cancer and therefore an increased risk for the disease. The risk conferred by family history is further increased if the affected family member was diagnosed with the disease at a young age. Pathogenic mutations in the *BRCA1* and *BRCA2* genes are responsible for the Hereditary Breast and Ovarian Cancer Syndrome (HBOC), but are present in less than 10% of women with breast cancer (Hulka and Moorman, 2001). Woman's hormonal history appears to be a risk factor, as the relative risk of breast cancer seems to

be related to the breast's cumulative exposure to estrogen and progesterone. Reproductive hormones stimulate cell division, thereby increasing the likelihood of DNA damage and the risk of cancer. Women who have had early menarche and late menopause (more menstrual cycles and thus greater hormone exposure) have a slightly higher risk of breast cancer (Kelsey and Bernstein, 1996). High breast tissue density (a mammographic indicator of the amount of breast and connective tissue relative to fatty tissue in the breast) has been shown to be a significant independent risk factor for breast cancer. In addition, mammographic detection of breast cancer is impaired in dense breast tissue (McCormack and dos Santos Silva, 2006). Breast cancer modifiable risk factors include body weight, physical activity, alcohol use, smoking, exposure to hormones (recent use of hormonal contraceptives, recent and long-term use of hormone replacement therapy), pregnancy, breastfeeding and radiation exposure (Hulka and Moorman, 2001).

Ovarian cancer risk rises with age and family cancer history, but tends to be reduced by factors which interrupt ovulation, such as pregnancy and breastfeeding (Edmondson and Monaghan, 2001). Oral contraceptive use confers long-term protection against ovarian cancer (Beral et al., 2008). Hormone replacement therapy increases the risk of ovarian cancer, although the magnitude is probably moderate (Garg et al., 1998; Beral et al., 2007). Women with endometriosis have an increased risk of developing ovarian cancer (Modugno et al., 2004). Also, women with a history of breast cancer have a twofold increased risk of ovarian cancer. The risk increases if their breast cancer is diagnosed before the age of 40 or if they have a family history of breast or ovarian cancer (Bergfeldt et al., 2002).

3. Breast and ovarian cancer pathology

Invasive breast carcinoma of no special type (NST), previously known as invasive ductal carcinoma not otherwise specified, is the most common form of breast cancer, accounting for 50-80% of all cases (Weigelt et al., 2008). Invasive lobular carcinoma is the second most common subtype. Additional subtypes of invasive breast carcinomas include tubular carcinoma, cribriform carcinoma, mucinous carcinoma, carcinoma with medullary features, carcinoma with apocrine differentiation, carcinoma with signet-ring cell differentiation, invasive

micropapillary carcinoma and metaplastic carcinoma of no special type. Precancerous lesions of the breast include both ductal carcinoma *in situ* (DCIS) and lobular neoplasia (Lakhani et al., 2012).

Ovarian tumors fall into three main groups – epithelial (approximately 60%), germ cell tumors (30%) and sex cord/stromal tumors (8%) – with rare miscellaneous entities outside these groups (Prat, 2015). Malignant epithelial tumors (carcinomas) are the most common ovarian cancers, accounting for 90% of the cases, and can be divided into five main types: high-grade serous (70%), endometrioid (10%), clear-cell (10%), mucinous (3%), and low-grade serous carcinomas (<5%) (Prat, 2012).

4. Hereditary breast and ovarian cancer syndrome

It is currently estimated that approximately 5-10% of all breast cancers are associated with inherited predisposition. There are three classes of breast cancer susceptibility genes (Figure 2): (1) high penetrance alleles (e.g., *BRCA1*, *BRCA2*, *TP53, PTEN, STK11, CDH1*), (2) rare moderate penetrance alleles (e.g. *CHEK2*, *ATM*, *PALB2*), and (3) common low penetrance alleles (e.g., *FGFR2*, *TOX3*) (Couch et al., 2014). Ovarian cancer susceptibility genes include *BRCA1*, *BRCA2*, *MLH1*, *MSH2*, *MSH6*, *BRIP1*, *RAD51C* and *RAD51D (Norquist et al., 2016)*. Although other inherited predisposition syndromes, like Li-Fraumeni syndrome, PTEN hamartoma tumor syndrome, Peutz–Jeghers syndrome, and Hereditary diffuse gastric cancer, are associated with high risk of breast cancer, HBOC is the most common Mendelian syndrome causing this disease.

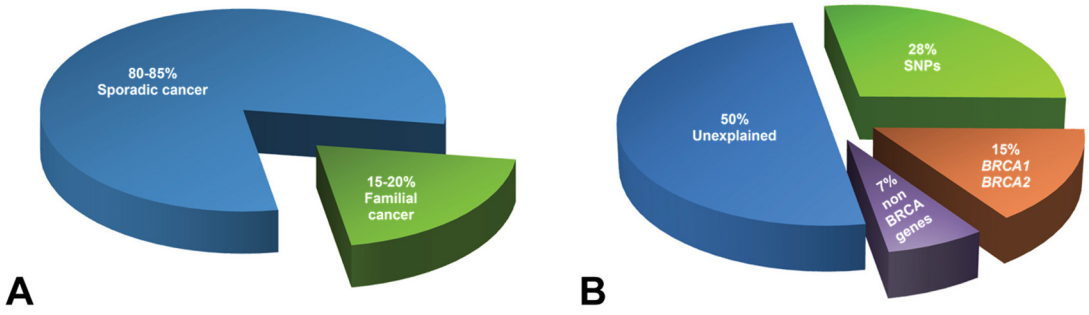

Figure 2 – A. Estimated percentage of sporadic and familial breast cancer patients. B. Estimated percentage contribution of genetic variants that predispose to breast cancer, namely, *BRCA1* and *BRCA2* genes, non *BRCA* genes (e.g., *TP53, PTEN, STK11, CDH1, CHEK2*, *ATM*, *PALB2*), and common low penetrance alleles (Single Nucleotide Polymorphisms, SNPs) (Couch et al., 2014).

Early descriptions of families with multiple cases of breast cancer date back centuries. The first comprehensive description was provided in 1866 by Paul Broca, a French surgeon, who was the first to describe a family with a high prevalence of carcinoma of the breast. His wife suffered from early onset breast cancer and her family showed four generations of breast cancer and occurrences of cancer of the gastrointestinal tract (Figure 3) (Lynch et al., 1972). This was the first report that pointed out that the risk of breast cancer can be inherited.

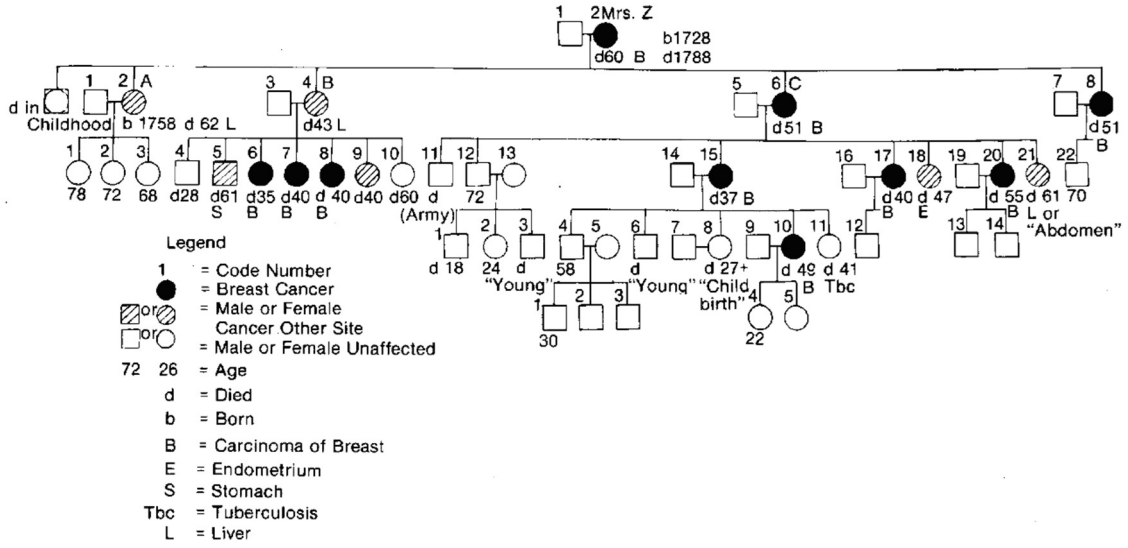

Figure 3 – Pedigree chart of Broca's family (Lynch et al., 1972).

The first description of the HBOC syndrome was done a century later by Lynch and coworkers, who described 34 families with two or more first-degree relatives affected with breast cancer in association with ovarian cancer and with a variety of other cancers (Lynch et al., 1972). Newman et al. (1988) analyzed data from 1579 families with multiple breast cancer cases and proposed an autosomal dominant model with a highly penetrant susceptibility allele, with incomplete penetrance that explained disease clustering (Newman et al., 1988).

In 1990, the genetic etiology for breast cancer predisposition was mapped to chromosome 17q21 (Hall et al., 1990). Narod and coworkers (1991) immediately confirmed linkage to the same *locus* to both breast and ovarian cancers in different families (Narod et al., 1991). In 1994, the *BRCA1* gene was cloned (Miki et al., 1994) and subsequently, in 1995, a second breast cancer gene, *BRCA2,* located on chromosome 13q12-13 was identified (Wooster et al., 1995).

INTRODUCTION

Up to 20% of women with invasive breast cancer report that one or more first degree relatives had a history of breast cancer (Collaborative Group on Hormonal Factors in Breast Cancer, 2001; National Institute for Health and Care Excellence (NICE) full guideline CG164, 2013). Pathogenic mutations in the *BRCA1* and *BRCA2* genes appear to account for 15% of familial breast cancers and for ~30% of high-risk breast cancer families (Figure 2) (Peto et al., 1999; Thompson and Easton, 2004; Couch et al., 2014). These families are often characterized by an early age of onset, overrepresentation of ovarian cancers, bilateral breast cancers, and male breast cancers. The mean age of breast cancer diagnosis is 39.9 years in *BRCA1* mutation carriers and 42.8 years in *BRCA2* mutation carriers, whereas for ovarian cancer it is 50.0 years in *BRCA1* mutation carriers and 54.5 years in *BRCA2* mutation carriers (Rebbeck et al., 2015).

BRCA1 and *BRCA2* are tumor suppressor genes (TSG) following the two-hit model: loss of heterozygosity (LOH) with loss of the wild type allele is frequently observed in BRCA tumor samples (Knudson, 1971; Collins et al., 1995; Cornelis et al., 1995). Disruption of both copies of the *BRCA1* or *BRCA2* genes in the mouse germ line caused early embryonic lethality (Liu et al., 1996; Friedman et al., 1998) and the specific inactivation of *Brca1* in mammary gland cells led to tumor development (Xu et al., 1999). However, data has been reported suggesting that loss of the BRCA wild-type allele is not required for carcinogenesis in several tissues and that, when LOH does occur, it is often a late event in disease progression (King et al., 2007; Venkitaraman, 2014).

4.1 Major breast and ovarian cancer susceptibility genes

4.1.1 The *BRCA1* gene

BRCA1 is a large gene that contains 23 exons, 22 of them coding, encoding an 1863 aminoacid protein with a predicted molecular mass of 207kDa. Exon 1 is non-coding and exon 11 encodes almost 60% of the full length BRCA1 protein. It should be noted that exon 4 does not exist owing to an error in the initial description of the gene (Miki et al., 1994). The genomic region encompassing *BRCA1* has an unusually high density of Alu repetitive DNA sequences (42%) (Welcsh and King, 2001). Many alternatively spliced transcript variants have been

described for this gene, although the functional relevance of the majority of them is still unknown (Colombo et al., 2014).

The *BRCA1* gene is ubiquitously expressed and it is responsible for the maintenance of genome stability mainly through its function in the repair of DNA double-strand breaks (DSBs) via the homologous recombination (HR) repair pathway (Venkitaraman, 2014). Additionally, BRCA1 is involved in multiple cellular functions, like transcription-coupled excision repair, chromatin remodeling, cell cycle regulation and ubiquitination (Narod and Foulkes, 2004; Roy et al., 2012). BRCA1 is also part of the BRCA1-associated genome-surveillance complex (BASC), which includes ATM, RAD50, MRE11, NBS1, and the mismatch repair proteins MLH1, PMS2, MSH2, and MSH6 (Wang et al., 2000).

The human *BRCA1* gene contains two highly conserved domains in the N- and C-terminal regions of the protein (Figure 4). At the N-terminus lies a RING (Really Interesting New Gene) domain (encoded by exons 2-7, aminoacids 1-109), which contains a series of eight conserved Cys3-His-Cys4 aminoacids and functions as an E3 ligase enzyme involved in ubiquitination (Clark et al., 2012). Two tandem repeat globular domains (exons 16-24, aminoacids 1650-1863), termed BRCA1 C-terminal (BRCT), a common feature of proteins involved in the DNA damage repair and cell cycle control, lie at the C-terminus (Clark et al., 2012).

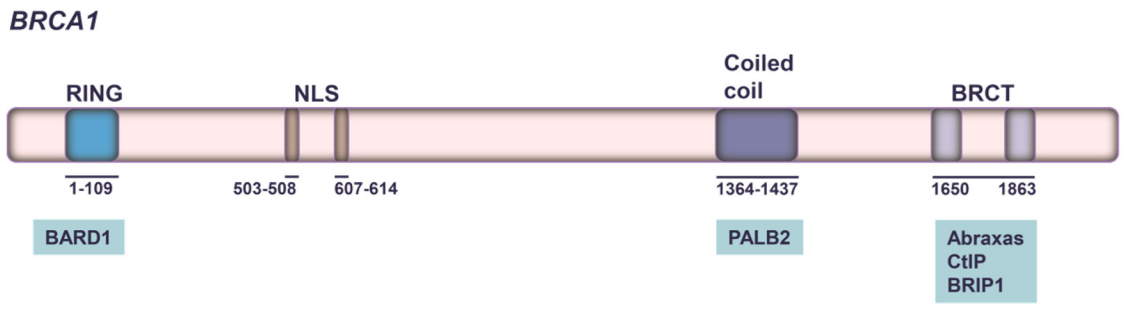

Figure 4 – *BRCA1* functional domains. At the N-terminus lies a RING domain (encoded by exons 2-7, aminoacids 1-109) and a Nuclear Localization signal (NLS) within the large central exon 11 (aminoacids 503-508 and 607-614). The C-terminus of BRCA1 contains a coiled-coil domain spanning exons 11-13 (aminoacids 1364-1437) that associates with PALB2, and a BRCA1 C-terminal (BRCT) domain (exons 16-24, aminoacids 1650-1863) that binds to Abraxas, CtIP and BRIP1 (Clark et al., 2012; Roy et al., 2012).

Sequences encompassing the RING domain are responsible for the interaction of BRCA1 with BARD1. BRCA1/BARD1 heterodimer has been implicated in the maintenance of genomic stability and tumor signaling, DNA repair, and transcriptional regulation (Roy et al., 2012). The ubiquitin ligase activity of BRCA1 is dramatically increased by formation of this heterodimer. Some mutations (e. g., p.Ile26Ala, p.Cys61Gly) in this domain are associated with predisposition to breast and ovarian cancer (Huen et al., 2010).

The BRCT domain is responsible for interactions with DNA damage repair proteins (Abraxas, BRIP1 and CtIP) that are phosphorylated by DNA damage-activated kinases, such as ATM and ATR (Huen et al., 2010). Both the RING and BRCT domains of the BRCA1 are very important for normal BRCA1 function and therefore a large number of breast cancer predisposing mutations are located in one of these two domains (Williams et al., 2004).

Additionally, two central nuclear localization signals (NLS) have been identified within the large central exon 11 (aminoacids 503-508 and 607-614). These domains are highly important for BRCA1 localization mediating BRCA1 transport from the cytosol to the nucleus. Mutation of the NLS sequences results in an altered cytosolic localization of BRCA1 (Clark et al., 2012).

A coiled-coil domain that mediates protein-protein interactions, spanning exons 11-13 of BRCA1 (aminoacids 1364-1437), contains the binding site for PALB2. PALB2 forms a complex together with BRCA1 and BRCA2 that is involved in HR during DNA repair (Clark et al., 2012). Mutations in the coiled-coil region of BRCA1 (e. g., p.Met1400Val, p.Leu1407Pro, and p.Met1411Thr) inhibit interaction between BRCA1 and PALB2 (Sy et al., 2009).

4.1.2 The *BRCA2* gene

The *BRCA2* gene (13q12-13) contains 27 exons, 26 of them coding, and encodes a 3418 aminoacid protein with a predicted molecular mass of 384kDa (Wooster et al., 1995). Like the *BRCA1* gene, exon 1 is non-coding and exon 11 is unusually large. BRCA2 is generally divided into three regions: the N-terminus, the BRC repeat region, and the C-terminal region containing a DNA Binding Domain (DBD) and an NLS domain (Figure 5). The N-terminus of BRCA2 binds to PALB2 at aminoacids 10-40 (Oliver et al., 2009), and this interaction was shown to be essential for loading RAD51 onto single-stranded DNA overhangs

of DSBs (Xia et al., 2006). The central region of the BRCA2 protein contains eight copies of the BRC repeat motifs of ~40 residues each, embedded in a ~1100-residue fragment within exon 11 (aminoacids 900-2000) that mediates binding to the RAD51 recombinase (Bork et al., 1996; Chen et al., 1998; Guidugli et al., 2014). BRCA2 mediates the recruitment of the recombinase RAD51 to DSBs at the appropriate sites of single-strand DNA (ssDNA) and promotes homologous pairing and strand invasion of these regions during HR. The C-terminal region (aminoacids 2459-3190) contains a DBD, which contains a helical domain (HD), three oligonucleotide binding (OB) folds that are ssDNA-binding modules, and a tower domain (TD) that mediates BRCA2 binding to double-stranded DNA (Guidugli et al., 2014). This domain also associates with deleted in split-hand/split-foot syndrome (DSS1), a small acidic protein that has been linked to BRCA2 protein stabilization (Yang et al., 2002).

The C-terminus of BRCA2 contains another RAD51 binding site (residues 3265-3330), which is phosphorylated by CDK1 that regulates BRCA2 interaction with RAD51 filaments (Davies and Pellegrini, 2007; Esashi et al., 2007). This region of BRCA2 also contains two NLS (aminoacids 3263-3269 and 3381-3385) that are important for the nuclear localization of BRCA2 (Spain et al., 1999; Guidugli et al., 2014).

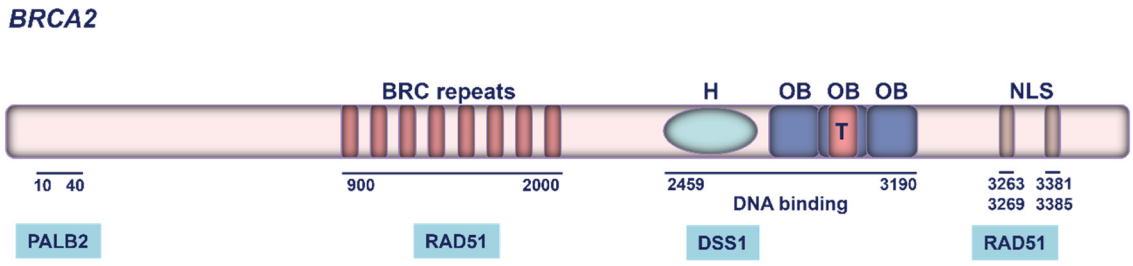

Figure 5 – Functional domains of the *BRCA2* gene. The N-terminus binds to PALB2 at aminoacids 10-40. The central region (within exon 11) contains eight copies of the BRC repeat motifs (aminoacids 900-2000), which mediates binding to the RAD51 recombinase. The C-terminal region (amino acids 2459-3190) contains a DNA Binding Domain (DBD), which contains a helical domain (HD), three oligonucleotide binding (OB) folds, and a tower domain (TD). This domain also associates with deleted in split-hand/split-foot syndrome (DSS1). The C-terminus of BRCA2 contains another RAD51 binding site (aminoacids 3265-3330) and two Nuclear Localization Signals (NLS) (aminoacids 3263-3269 and 3381-3385) (Roy et al., 2012; Guidugli et al., 2014)

4.1.3 Involvement of BRCA1 and BRCA2 in DNA repair

The *BRCA* genes are involved in the preservation of genome integrity through their role in DNA damage signaling and DNA repair. Their inactivation promotes chromosomal instability, leading to an increased frequency of alterations in other genes, so they are considered caretaker TSGs. A caretaker gene does not directly regulate cellular proliferation by inhibiting growth or promoting cell death, but their inactivation destabilizes the genome and affects gatekeeper genes, which then leads to tumor formation.

BRCA1- and BRCA2-deficient cells have similar cellular phenotypes, including structural aberrations that include breaks affecting a single sister chromatid, as well as quadriradial and triradial chromosomes. These structural anomalies are accompanied by aberrations in chromosome number reflecting inaccurate chromosome segregation (Patel et al., 1998; Shen et al., 1998; Yu et al., 2000). Structural chromosomal aberrations following *BRCA* inactivation are believed to arise from their role in DNA repair by HR. Homologous recombination is an error free mechanism that repairs DSBs. BRCA1 and BRCA2 are individually essential for an efficient HR in mammalian cells and in their absence replication associated DNA breaks can instead be repaired by error-prone mechanisms, like nonhomologous end joining (NHEJ) (Huen et al., 2010; Liu et al., 2014).

BRCA1 is directly involved in HR-mediated repair of DSBs. In response to a DSB, the damage is detected by the “MRN” complex (MRE11-RAD50-NBS1), a DNA damage sensor, which is recruited to DNA broken ends (Lee and Paull, 2005). MRN recruits and activates ATM, a nuclear protein kinase, which then phosphorylates the variant histone H2AX (Burma et al., 2001). Several BRCA1 protein complexes are then recruited to DSB ends, where they regulate the resection process and also engage the G2/M cell cycle checkpoint (activation of the ATR/ChK1 pathway) responsible for S phase arrest, thereby preventing the replication of damaged DNA (Rosen, 2013). 5´ends of the broken DSB ends are resected, producing long stretches of 3’ ssDNA on either side of the DSB. Replication protein A (RPA) binds to the ssDNA in order to prevent the formation of secondary DNA structures and avoid nuclease digestion. After resection, another BRCA1 complex (BRCA1/BRCA2/ PALB2/RAD51) promotes the exchange of RPA for RAD51. The recruitment of the recombinase RAD51 to

DSBs is mediated by BRCA2 BRC repeats. The BRC repeats promote nucleoprotein filament formation by stabilizing RAD51-ssDNA interactions while inhibiting RAD51-double strand DNA binding. The RAD51-ssDNA filament subsequently mediates sister chromatid strand invasion, promoting DNA pairing between homologous sequences resulting in an error free repair (Figure 6) (Venkitaraman, 2014)

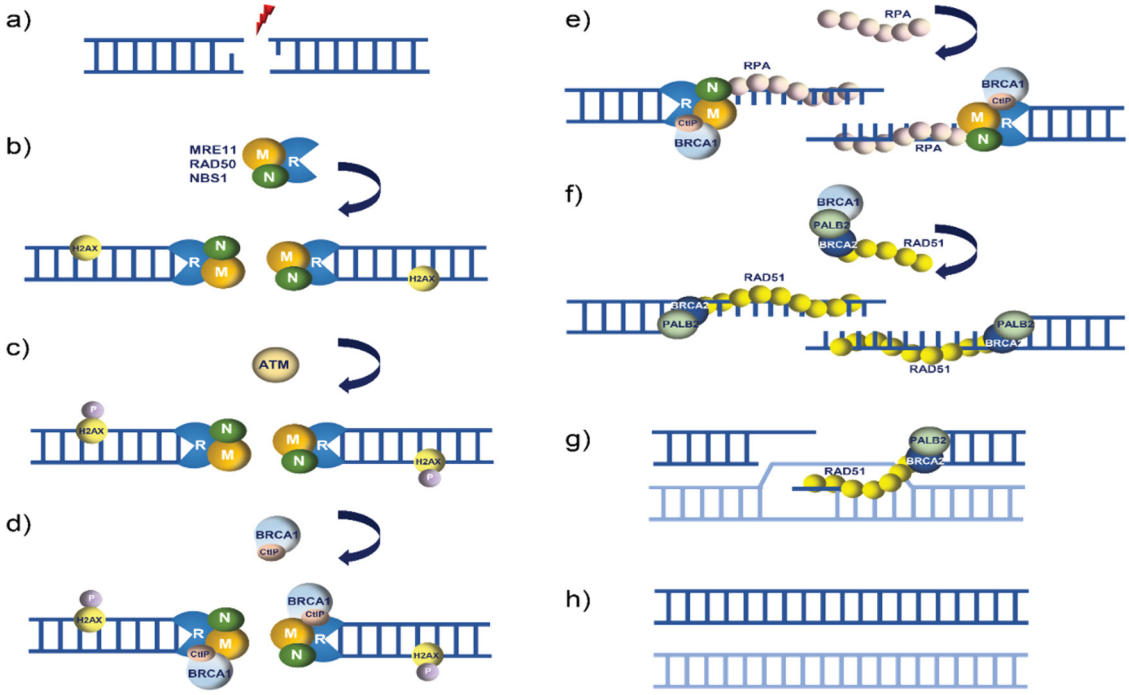

Figure 6 – BRCA1 and BRCA2 mediated homologous recombination repair. In response to DNA Double-Strand Breaks (DSBs) (a), the MRN complex (MRE11-RAD50-NBS1) is recruited (b) and activates ATM, which phosphorylates histone H2AX (c). Several BRCA1 protein complexes (*e.g.* BRCA1/CtIP complex) are then recruited to DSB ends, producing long stretches of 3' single-strand DNA (ssDNA) (d). Replication protein A (RPA) binds to the ssDNA (e) and, after resection, another BRCA1 complex (BRCA1/BRCA2/PALB2/RAD51) promotes the exchange of RPA for RAD51 (f). Homologous recombination occurs through sister chromatid exchange, resulting in an error free repair (g, h) (Venkitaraman, 2014).

4.1.4 Tissue specificity of cancer predisposition

Several hypotheses have been proposed to address the tissue specific cancer risk, in particular that of the breast and ovary, associated with *BRCA1* and *BRCA2* mutations. Given that both breast and ovary are estrogen-responsive tissues, Scully and Livingston (2000) hypothesized that DNA adducts formed by some estrogen metabolites could not be repaired in BRCA disrupted cells (Scully and Livingston, 2000). Elledge and Amon (2002) proposed the hypothesis that loss of *BRCA1* leads in most tissues to cell cycle arrest and, if DNA damage is

not successfully repaired, to apoptosis, whereas in the breast and ovary *BRCA1*-negative cells receive the necessary growth and survival factors (hormones, growth factors, *etc*.) and continue to divide (Elledge and Amon, 2002). This increases the possibility that secondary mutations accumulate over time (such as TP53 inactivation), which in some cases will lead to the development of breast and/or ovarian cancer. Another model for BRCA tissue specific carcinogenesis has been proposed and combines the responsiveness of breast and ovary tissues to estrogen and the genomic context of the *BRCA* genes. During puberty and pregnancy breast epithelial cells rapidly proliferate due to increased levels of estrogen. It is likely that, during these periods, the repair capacity of these cells is compromised and somatic alterations mediated by the high densities of repetitive elements of the *BRCA* genes will occur at high frequency, leading to the loss of a *BRCA1* and *BRCA2* allele (LOH) (Welcsh and King, 2001). Tissue-specific expression of genes that interact with BRCA1 and BRCA2 may play a role as well (Welcsh et al., 2000).

4.1.5 *BRCA1* and *BRCA2* mutation prevalence and penetrance

Prevalence of *BRCA* mutations refers to its frequency in the population. The prevalence of *BRCA* disease-causing mutations has been estimated and can vary in different populations, being 0.2 to 0.3% in unselected women and 6 (breast or ovarian cancer onset ≤ 40 years) to 19.8% (family history of breast or ovarian cancer) in high-risk populations (Nelson et al., 2013).

Penetrance is the probability of developing the phenotype in carriers and is reported as the cumulative risk until a specified age. HBOC syndrome is an autosomal dominant disease with incomplete penetrance. Pathogenic mutations in the *BRCA1* and *BRCA2* genes confer high risk for breast and ovarian cancer. Other cancers consistently associated with *BRCA1* mutations include prostate cancer, male breast cancer, and melanoma (Liede et al., 2004; Leongamornlert et al., 2012; Mersch et al., 2015). *BRCA2* mutations are associated with prostate cancer, male breast cancer, pancreatic cancer and melanoma (Breast Cancer Linkage Consortium, 1999; Liede et al., 2004; Mersch et al., 2015). The likelihood that a *BRCA* mutation carrier will develop a cancer (risk estimate) varies with the ascertainment criteria, with higher estimates derived from studies of selected high-risk families (Easton et al., 1993; Ford et al., 1994; Easton et al., 1995; Ford

et al., 1998). The lifetime risk for female breast cancer in *BRCA1* and *BRCA2* mutation carriers is estimated to be as high as 85%, whereas the lifetime risk of ovarian cancer is estimated to be 40 to 60% for *BRCA1* carriers and 15 to 25% for *BRCA2* carriers (Easton et al., 1993; Ford et al., 1994; Easton et al., 1995; Ford et al., 1998). Lower penetrance estimates have been found in population-based studies (Antoniou et al., 2003; Chen and Parmigiani, 2007; Nelson et al., 2013). A recent study indicates that *BRCA1* mutation carriers have, by age 70, an estimated breast cancer risk of 46-70% and an estimated ovarian cancer risk of 41-46%, whereas *BRCA2* mutation carriers are estimated to have a 50-71% breast cancer risk and a 17-23% ovarian cancer risk (Nelson et al., 2013).

Risk estimates vary between studies not only because of different ascertainment used in the penetrance estimation (e.g. population based versus families with multiple affected individuals), but also because risk estimates vary by age at diagnosis of the proband that led to the family ascertainment (young onset *versus* older cancer cases), type and site of the cancer (e.g. unilateral versus bilateral breast cancer, breast *versus* ovarian cancer), mutation type or position within the gene (Antoniou et al., 2003; Begg et al., 2008; Rebbeck et al., 2015), and genetic modifiers of *BRCA1* and *BRCA2* genes (Antoniou et al., 2008; Friebel et al., 2014). Several studies evaluated if established risk factors for breast and ovarian cancer in the general population are also risk factors in *BRCA* mutation carriers, but it remains unclear which risk factors may be used in risk counseling or lifestyle modification to minimize cancer risk (Pruthi et al., 2010; Friebel et al., 2014).

4.1.6 Histopathology and molecular features of *BRCA1* and *BRCA2* tumors

Breast tumors express a number of immunohistochemical markers, namely estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2), which provide both prognostic and predictive information. Among sporadic tumors, 70% are ER-positive and 50% are PR-positive, and HER2-overexpression is observed in approximately 15% of the cases. Approximately 20% of all breast cancer cases are negative for ER, PR, and HER2, being known as “triple-negative” (TN) cancers. The prognosis of TN tumors is poor, not only because these tumors seem to be more aggressive,

but also because hormonal therapy and anti-HER2 therapies are ineffective in this breast cancer subtype (Honrado et al., 2004; de Ruijter et al., 2011).

Breast tumors arising in carriers of *BRCA1* or *BRCA2* mutations display characteristic pathological features. Most hereditary breast cancers are invasive carcinomas (NST), but *BRCA1* related tumors have more frequently medullary features like pushing margins, lymphocytic infiltrate, and higher mitotic count than sporadic controls (Honrado et al., 2004). *BRCA1* tumors have been found to be more frequently ER, PR, and HER2 negative (Mavaddat et al., 2012) and hence typically display the TN phenotype. They often express basal markers, such as cytokeratins 5/6 and cytokeratin 14, and are included in the 'basal' subtype in gene expression studies (Sorlie et al., 2003). Higher frequencies of *TP53* mutations have been described in *BRCA1* carcinomas (Greenblatt et al., 2001).

Similar to *BRCA1*-related breast cancers, the most common histological type in *BRCA2* tumors is invasive breast carcinoma (NST) (76%) (Breast Cancer Linkage Consortium, 1997). Although a higher incidence of tubular and lobular carcinomas has been reported for *BRCA2* tumors (Marcus et al., 1997; Mavaddat et al., 2012), other studies have not confirmed these observations (Lakhani et al., 1998). Most *BRCA2* tumors are grade 2/3 with high mitotic rates. Continuous pushing margins are also characteristic of *BRCA2* tumors. In contrast to *BRCA1* tumors, *BRCA2* tumors seem to be more similar to sporadic tumors with relation to the expression of ER and PR (Lakhani et al., 2002). *BRCA2* tumors rarely overexpress or show amplification of HER2 (Mavaddat et al., 2012).

Most ovarian cancers from *BRCA1* and *BRCA2* mutation carriers are invasive epithelial cancers of serous histology and have higher grade compared with sporadic ovarian cancers (Lakhani et al., 2004). Approximately 13-17% of high grade ovarian cancer is attributable to germline mutations in *BRCA1* or *BRCA2* (Pal et al., 2005; Cancer Genome Atlas Research Network, 2011). There are no significant differences in ovarian cancer morphology and grade between *BRCA1* and *BRCA2* mutation carriers (serous: 67%; endometrioid: 12%; clear-cell: 2%; mucinous: 1%) (Mavaddat et al., 2012). It is now widely accepted that most high grade serous ovarian carcinomas develop from the neoplastic progression of epithelial cells of the fallopian tube (Crum et al., 2007; Kindelberger et al., 2007). Several findings support this hypothesis, namely: the absence of precursor lesions identified in the ovary with high grade serous cancer

(Piek et al., 2001; Yates et al., 2011); gene expression studies showing that serous ovarian cancers are more similar to tubal epithelium rather than ovarian epithelium (Crum et al., 2007); and the reduced risk for serous ovarian cancer after tubal ligation in the general population (Crum et al., 2007) and *BRCA* carriers (Antoniou et al., 2009; Walker et al., 2015).

BRCA1 and *BRCA2* breast tumors have a higher frequency of *TP53* mutations than sporadic cancers (Greenblatt et al., 2001). Somatic loss of *PTEN* (intragenic chromosome breaks, inversions, deletions, intragenic copy number aberrations) is also associated with *BRCA1* tumors (Saal et al., 2008). Comparative genomic hybridization analysis revealed that BRCA breast tumors contain a large number of chromosomal copy number gains and losses comparative to controls. The genetic changes more commonly found in *BRCA1* tumors are gain of 8q and 3q and losses of 4p, 4q and 5q. *BRCA2* tumors present gains of 8q, 17q and 20q and losses of 8p, 13q and 11q (Tirkkonen et al., 1997; Wessels et al., 2002; Jonsson et al., 2005).

High-grade serous ovarian carcinomas, including *BRCA1* and *BRCA2* tumors, have a high rate of *TP53* mutations (96%) (Cancer Genome Atlas Research Network, 2011). Overall, hereditary and sporadic epithelial ovarian cancer exhibit a similar pattern of somatic alterations, showing regions of recurrent gains at 3q26 and 8q24 and losses at 8p23 and 17p (Kamieniak et al., 2013). However, *BRCA1* and *BRCA2* ovarian carcinomas have on average a higher number of losses and homozygous deletions, while sporadic cases present a higher number of gains and amplifications (Kamieniak et al., 2013).

4.2 Identification of HBOC families

4.2.1 Risk assessment

Screening for inherited breast and ovarian cancer susceptibility includes assessment of the *a priori* probability of *BRCA* mutations, followed by genetic testing of high-risk individuals. Genetic testing criteria for *BRCA1* and *BRCA2* may differ between countries based on mutation prevalence and the existence of founder mutations. However, widely accepted clinical criteria for further personalized risk assessment, genetic counseling and often genetic testing are recommended by the National Comprehensive Cancer Network (NCCN) panel (NCCN Version 2.2016, 2016), including personal and family history of breast,

ovarian, prostate and pancreatic cancer, age at diagnosis, Ashkenazi Jewish ancestry and previously identified *BRCA1*/*BRCA2* pathogenic mutation in the family.

National Institute for Health and Care Excellence (NICE) guidelines recommend offering genetic testing when there is a 10% or more likelihood of carrying a *BRCA1/2* mutation based on predictive models such as BRCAPRO, BOADICEA or Manchester Score (National Institute for Health and Care Excellence (NICE) full guideline CG164, 2013). Genetic testing should be performed in adults after they have received genetic counseling and given informed consent.

4.2.2 HBOC syndrome management

The effectiveness of breast self-examination has not been formally evaluated in women with a hereditary risk for breast cancer. Mammography efficacy in detecting breast tumors in high-risk women is limited due not only to the higher breast density in young women but also to the propensity for the development of more aggressive and rapidly growing cancers in these women (Gilliland et al., 2000; Tilanus-Linthorst et al., 2002). Magnetic resonance imaging (MRI) is more sensitive than mammography in detecting tumors in women with a *BRCA1*/*2* mutation, but has a lower specificity (Warner et al., 2004).

Current breast cancer screening recommendations for female carriers of a *BRCA1*/*2* mutation include monthly breast self-exam beginning at 18 years of age and semiannual clinical breast exam beginning at age 25 years (NCCN Version 2.2016, 2016). Although screening strategies are still under study, the recommendations are annual breast MRI screening (preferred) or mammogram if MRI is unavailable between 25-29 years (or individualized based on family history, if breast cancer diagnosis before age 30 is present) and annual mammogram and breast MRI screening between 30-75 years. For women older than 75 years, management should be considered on an individual basis. Screening should be modified based on the earliest age of onset of breast cancer in a first-degree relative in a given family. Although surveillance methods for early detection of ovarian tumors (transvaginal ultrasounds and CA-125) have not been

shown to be an effective strategy, transvaginal ultrasounds may be considered starting at age 30-35 years (NCCN Version 2.2016, 2016).

Men positive for a *BRCA1* or *BRCA2* mutation should be followed with clinical breast self-exam training and education starting at age 35 years and annual clinical breast exam beginning at age 35 years. Prostate cancer screening is recommended for *BRCA2* carriers starting at age 40 years and should also be considered for *BRCA1* carriers (NCCN Version 2.2016, 2016).

For both men and women carriers of *BRCA1/2* mutations, a full body skin exam for melanoma screening and investigational protocols for pancreatic cancer screening should be considered (NCCN Version 2.2016, 2016).

Prophylactic mastectomy (PM) and prophylactic bilateral salpingo-oophorectomy (PBSO) are effective options for reducing breast cancer risk in women carriers of *BRCA1*/*2* mutations, with PM reducing the risk by at least 85% and PBSO reducing the risk by 50% (if performed before menopause). Additionally, PBSO reduces ovarian cancer risk by 69% to 100% (Nelson et al., 2013; Heemskerk-Gerritsen et al., 2015). The NCCN Guidelines panel supports discussion of the option of risk reduction mastectomy for women on a case-by-case basis. Counseling regarding the degree of protection offered by such surgery and the degree of cancer risk should be provided. Bilateral total simple mastectomy provides a more complete removal of breast tissue than the previously used subcutaneous mastectomy. However, none of the procedures completely removes all breast tissue and breast cancer can still occur postmastectomy (Rebbeck et al., 2004). Additionally, the NCCN guidelines panel recommends that women should consider salpingo-oophorectomy (between 35 and 40 years, and upon completion of child bearing) as an effective risk-reduction strategy given the absence of reliable methods of early detection and the poor prognosis associated with advanced ovarian cancer (NCCN Version 2.2016, 2016). In patients with *BRCA2* mutations who already undergone bilateral mastectomy it is reasonable to delay RRSO until age 40-45 years because ovarian cancer onset in those women is on average 8-10 years later than in patients with *BRCA1* mutations. After bilateral oophorectomy the occurrence of peritoneal carcinomatosis remains a possibility (Rebbeck, 2002). Also, ovarian cancer risk reduction might theoretically be attained by a bilateral salpingectomy in younger women until the age of natural menopause, when a bilateral

oophorectomy could be performed (Greene et al., 2011). Concerns for risk-reducing salpingectomy (without oophorectomy) include the fact that women are still at risk for developing ovarian cancer. Moreover, women who opt to delay oophorectomy will not benefit from the 50% reduction in breast cancer provided through premenopausal oophorectomy (Society of Gynecologic Oncology, 2013).

Tamoxifen and raloxifene, which are selective ER modulators (SERMs), reduce risk for breast cancer in women at high risk (Vogel, 2010), although only limited data are available in patients with *BRCA* mutations. The efficacy of SERMs in specifically reducing the incidence of breast cancer in healthy *BRCA1*/*2* mutation carriers has not been elucidated. Although the limited data indicated that tamoxifen may be more effective in reducing breast cancer risk in *BRCA2* mutation carriers when compared to *BRCA1* carriers (King et al., 2001), a case-control study of *BRCA1*/*2* mutation carriers with breast cancer demonstrated a protective effect of this drug against contralateral breast cancer in both *BRCA1*/*2* mutation carriers (Gronwald et al., 2006). On the other hand, oral contraceptives have been shown to reduce ovarian cancer risk in the general population and also in *BRCA1*/*2* mutation carriers (Beral et al., 2008; Iodice et al., 2010).

4.2.3 Prognosis of patients with HBOC syndrome

There are contradictory results regarding breast cancer prognosis in women with *BRCA1*/*2* germline mutations compared with those with sporadic breast cancer. Given the more adverse tumor characteristics in BRCA-associated cancers, it was suggested that breast cancer prognosis may differ in women with germline mutations in these genes compared with those with sporadic breast cancer (Goodwin et al., 2012). However, several recent studies that have investigated breast cancer prognosis in women with *BRCA* mutations failed to demonstrate that *BRCA1*/*2* mutation carriers have worse breast cancer survival (Rennert et al., 2007; Bordeleau et al., 2010; van den Broek et al., 2015).

Epithelial ovarian cancer patients with germline *BRCA1* or *BRCA2* mutations have an improved survival compared to non carriers, with *BRCA2* carriers having the best prognosis (Bolton et al., 2012).

Treatment of these tumors influences prognosis because *BRCA1* and *BRCA2* genes are involved in DNA repair, rendering these tumors more sensitive

to chemotherapy that causes DNA breaks (e.g., alkylating agents, platinum) (Goodwin et al., 2012).

4.2.4 Targeted treatment

The BRCA proteins are essential for maintenance of chromosome integrity, offering the possibility for new therapeutic approaches. Chemotherapies that arrest replication, such as DNA cross-linking agents (platinum compounds), are particularly effective against *BRCA1* (and, by analogy, *BRCA2*) mutant tumors (Byrski et al., 2009; Byrski et al., 2010). Platinum compounds generate interstrand cross-links that, in the absence of the BRCA proteins, cannot be repaired by HR. In addition, inhibitors of the enzyme poly (ADP-ribose) polymerase 1 (PARP1), an enzyme that is involved in base excision repair pathway (BER), are particularly effective in BRCA defective cancer cells. PARP1 inhibition leads to spontaneous ssDNA lesions and during DNA replication these DNA lesions can degenerate to form DSBs that activate HR repair. However, in BRCA deficient cells the HR DNA repair pathway is impaired, sensitizing the cancer cells to PARP1 inhibition. Disabling both the BER and the BRCA-dependent pathways (synthetic lethality) results in cell death due to chromosomal instability, cell cycle arrest, and apoptosis (Bryant et al., 2005; Farmer et al., 2005; Domchek et al., 2011). Synthetic lethality refers to the mutation or inhibition of two genes or molecular pathways that leads to cell death when inhibition of either one alone would not (Domchek et al., 2011). Recently, a PARP inhibitor (olaparib) developed for the treatment of solid tumors was approved in Europe and in the United States of America (USA) for the treatment of BRCA-mutated ovarian cancer and clinical trials are ongoing in other BRCA-associated tumor types (Deeks, 2015).

4.3 Germline *BRCA1* and *BRCA2* mutation spectrum

More than 3700 distinct variants have been reported in the *BRCA1* and *BRCA2* genes, including pathogenic mutations, polymorphisms and variants of unknown significance, of which 2030 have been reported only once (BIC database, 2016). Most of the putative pathogenic mutations found in these genes are either frameshift or nonsense mutations that theoretically cause the production of truncated proteins. They usually cause loss of important functional

domains or result in unstable transcripts and therefore can be classified with reasonable confidence as loss-of-function mutations. In contrast, the functional impact of most missense, silent, and intronic variants or in-frame deletions and insertions is unknown. Only a few missense mutations could be demonstrated to be disease causing (Huen et al., 2010; Guidugli et al., 2013; Guidugli et al., 2014). Furthermore, large gene rearrangements (LGRs) have been identified in HBOC families, most being related with the high content of Alu elements in the intronic sequences of both *BRCA1* and *BRCA2* genes (Welcsh and King, 2001). These genomic rearrangements include primarily deletions and duplications of one or more exons and represent 10-15% of all deleterious germline mutations in the *BRCA1* gene and 1-7% in the *BRCA2* gene (Mazoyer, 2005; Sluiter and van Rensburg, 2011). Rearrangements with distinct breakpoints but involving the same exons may be described similarly, making it difficult to assess whether a given rearrangement is new or even specific of a given population (van den Ouweland et al., 2009). To determine whether a LGR is actually novel and to gain insight about the mutational mechanism responsible for its occurrence, molecular characterization with breakpoint identification is mandatory.

4.3.1 Founder mutations

Molecular analyses of the *BRCA1* and *BRCA2* genes in families suspected to have hereditary predisposition to breast/ovarian cancer have shown that most populations exhibit a wide spectrum of mutations throughout both genes. However, several founder mutations have been identified in individuals of different ancestries, including those of Ashkenazi Jewish (185delAG and 5382insC) (Tonin et al., 1995), French Canadian (C4446T and 2953del3+C) (Tonin et al., 1998), Norwegian (1675delA and 1135insA) (Dorum et al., 1999), Dutch (2804delAA and Alu-mediated deletions encompassing exons 13 and 22) (Peelen et al., 1996), Polish (5382insC, C61G, and 4153delA) (Gorski et al., 2004), Greek (5382insC and G1738R) (Konstantopoulou et al., 2008), Icelandic (999del5) (Rafnar et al., 2004), Spanish (330A>G, 6857_6858del, and 9254_9258del) (Diez et al., 2003), and Swedish (3171ins5) (Bergman et al., 2001) origins. Also, the contribution of LGRs to *BRCA1*/*2* mutation-positive families varies in a population-dependent manner and numerous *BRCA1* founder rearrangements have been identified in particular ethnic groups (Petrij-Bosch et

al., 1997; Hogervorst et al., 2003; Weitzel et al., 2007). Identification of founder mutations makes it possible to use more specific approaches to molecular testing (Tonin et al., 1998), 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 the identification of genetic and environmental risk modifiers.

4.3.2 Mutation pattern in Portuguese HBOC families

The first characterization of the mutational spectrum of the entire coding sequences and exon-intron boundaries of the *BRCA1* and *BRCA2* genes, as well as large *BRCA1* rearrangements, in Portuguese families with inherited predisposition to breast/ovarian cancer identified a nonrandom spectrum of germline *BRCA1*/*BRCA2* mutations (Peixoto et al., 2006). Inherited cancer predisposition could be linked to *BRCA1* and *BRCA2* mutations in 22 of the 100 probands (and in 24.7% of those with a family history of cancer). A regional founder effect was subsequently described for a *BRCA2* rearrangement (c.156_157insAlu) in hereditary breast/ovarian cancer families mostly originated from central/southern Portugal (Machado et al., 2007). This rearrangement was first identified in a Portuguese breast cancer patient living in Belgium, and corresponds to an Alu insertion in *BRCA2* exon 3 that is not detectable using the conventional mutation screening techniques (Teugels et al., 2005). Teugels et al. (2005) demonstrated that the c.156_157insAlu mutation originates an in-frame deletion of exon 3 from the *BRCA2* mRNA encoding a transcriptional activation domain (Teugels et al., 2005). However, some doubts have been raised about the pathogenic effect of *BRCA2* exon 3 skipping (Diez et al., 2007).

Recently, several *BRCA1*/*BRCA2* variants of uncertain significance (VUS), identified in Portuguese families fulfilling the criteria for germline mutation screening, were studied in order to ascertain their pathogenicity (Santos et al., 2014). Of the 25 variants analyzed, four were classified as pathogenic mutations, 16 as neutral variants, three as polymorphisms and only two remained unclassified variants. These findings are helpful for adequate genetic counseling of the families carrying those variants, especially in those that are deleterious, because it becomes possible to offer predictive testing to family members at risk,

therefore allowing adoption of prophylactic measures and/or rigorous surveillance of the carriers and reducing the anxiety of the noncarriers (Santos et al., 2014).

To determine the best strategy for molecular testing of Portuguese breast/ovarian cancer families, it is very important to identify the type, the mechanism of pathogenicity and the frequency of *BRCA1* and *BRCA2* germline mutations in breast/ovarian cancer families (Peixoto et al., 2006; Santos et al., 2009; Santos et al., 2014). Identification of frequent (recurrent and/or founder) mutations is an extremely important step to increase the efficiency of genetic testing, since it makes possible the use of more specific, cheaper and quicker strategies to identify HBOC families. On the other hand, the existence of founder mutations may also allow widening the criteria for genetic testing, potentially enabling the diagnosis of HBOC patients with an *a priori* mutation probability below the commonly used testing threshold of 10%. However, the criteria for genetic testing of Portuguese founder mutations have not been established.

AIMS

This study aimed to characterize the spectrum of *BRCA1*/*2* germline mutations in Portuguese HBOC families, including the identification of founder mutations. Specifically, the objectives of this thesis were:

1. To evaluate the contribution of the c.156_157insAlu *BRCA2* mutation to inherited predisposition to breast/ovarian cancer in families originated mostly from northern/central Portugal.
2. To gain insight into the ancestral origin and population spread of the c.156_157insAlu *BRCA2* mutation.
3. To characterize the pathological and clinical significance of the c.156_157insAlu *BRCA2* mutation.
4. To perform molecular characterization, with breakpoint identification, of LGRs detected in Portuguese HBOC families.
5. To characterize the mutational spectrum and to evaluate the impact of founder mutations in the genetic testing criteria and strategy for molecular testing of HBOC families of Portuguese ancestry.

PAPER I

Ana Peixoto, Catarina Santos, Patrícia Rocha, Manuela Pinheiro, Sofia Príncipe, Deolinda Pereira, Helena Rodrigues, Fernando Castro, Joaquim Abreu, Leonor Gusmão, António Amorim, Manuel R. Teixeira

"The c.156_157insAlu *BRCA2* rearrangement accounts for more than one-fourth of deleterious BRCA mutations in northern/central Portugal"

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PRECLINICAL STUDY

The c.156_157insAlu *BRCA2* rearrangement accounts for more than one-fourth of deleterious BRCA mutations in northern/central Portugal

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Abstract We evaluated the contribution of an Alu insertion in *BRCA2* exon 3 (c.156_157insAlu) to inherited predisposition to breast/ovarian cancer in 208 families originated mostly from northern/central Portugal. We identified the c.156_157insAlu *BRCA2* mutation in 14 families and showed that it accounts for more that one-fourth of deleterious *BRCA1*/*BRCA2* mutations in breast/ovarian cancer families originated from this part of the country. This mutation originates *BRCA2* exon 3 skipping and we demonstrated its pathogenic effect by showing that the *BRCA2* full length transcript is derived only from the wild type allele in carriers, that it is absent in 262 chromosomes from healthy blood donors, and that it co-segregates with the disease. Polymorphic microsatellite markers were used for haplotype analysis in three informative families. In two of the three families one haplotype was shared for all but two markers, whereas in the third family all markers telomeric to *BRCA2* differed from that observed in the other two. Although the c.156_157insAlu *BRCA2* mutation has so far only been identified in Portuguese breast/ovarian cancer families, screening of this rearrangement in other populations will allow evaluation of whether or not it is a population-specific founder mutation and a more accurate estimation of its distribution and age.

Keywords c.156_157insAlu *BRCA2* mutation · Breast cancer · Hereditary predisposition

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Introduction

Molecular analyses of the *BRCA1* and *BRCA2* genes in families suspected to have hereditary predisposition to breast/ovarian cancer have shown that most populations exhibit a wide spectrum of mutations throughout both genes. However, several founder mutations have been identified in individuals of different ancestries, including those of Ashkenazi Jewish (185delAG and 53832insC) [1], French Canadian (C4446T and 2953del3+C) [2, 3], Norwegian (1675delA and 1135insA) [4], Dutch (2804delAA and Alu-mediated deletions encompassing exons 13 and 22) [5, 6], Polish (5382insC, C61G, and 4153delA) [7], Greek (5382insC and G1738R) [8, 9], Icelandic (999del5) [10], Spanish (330A>G, 6857_6858del, and 9254_9258del) [11, 12], and Swedish (3171ins5) [13] origins. We have previously reported several novel mutations in a first characterization of the mutational spectrum of the entire coding sequences and exon–intron boundaries of the

BRCA1 and *BRCA2* genes and large *BRCA1* rearrangements in Portuguese breast/ovarian cancer families [14].

Recently, Teugels et al. [15] identified an Alu insertion in *BRCA2* exon 3 (c.156_157insAlu) in a Portuguese breast cancer patient living in Belgium. This mutation was not detectable using the conventional mutation screening techniques and originates skipping of *BRCA2* exon 3 [15]. A regional founder effect has recently been described for this rearrangement in hereditary breast/ovarian cancer families mostly originated from central/southern Portugal [16]. In an attempt to gain insight into the ancestral origin and population spread of the c.156_157insAlu *BRCA2* mutation, we estimated its frequency in breast/ovarian cancer families originated mostly from northern/central Portugal and analyzed the chromosomal background upon which this mutation occurs using 10 microsatellite markers surrounding the *BRCA2* gene. Furthermore, because some doubts have been raised about the pathogenic effect of *BRCA2* exon 3 skipping [17], we have performed segregation studies and mRNA expression analysis of c.156_157insAlu carriers and disease-free controls.

Materials and methods

Patients

This study comprised the 78 Portuguese breast/ovarian cancer families in whom no clearly deleterious *BRCA1*/*BRCA2* mutations were found in our previous study [14] and an additional 130 *BRCA1*/*BRCA2* negative probands that had been selected using the following the criteria: (1) families with one or more breast cancers (one of them diagnosed before the age of 50 years) and one or more ovarian cancers (BC + OC); (2) families with two or more female breast cancers, one of them diagnosed before the age of 50 years (≥2BC); (3) families with at least one case of breast cancer or ovarian cancer in association with at least one case of male breast cancer (MBC); (4) patients without family history of breast/ovarian cancer, but showing early-onset breast cancer (1BC, diagnosed before the age of 35 years), breast (diagnosed before the age of 60 years) and ovarian cancer (1BC + OC), bilateral breast carcinomas (1BlC, one of them diagnosed before the age of 50 years), or male breast cancer (1MBC, at any age). BRCAPRO mutation probabilities were estimated for all probands [18].

DNA isolation and *BRCA1*/*BRCA2* mutation screening

Genomic DNA was extracted from peripheral leucocytes according to standard procedures and subsequently amplified using primers described by van der Hout et al. [19]. Mutation screening of the entire coding regions of *BRCA1* and *BRCA2* was performed by Denaturing Gradient Gel Electrophoresis (DGGE), using the IngenyphorU system (Ingeny; Goes). Sequence analysis of genomic fragments with altered DGGE mobility pattern was carried out on an Applied Biosystems' 310 automated DNA sequencer (Foster City), using the dye terminator method. Multiplex Ligation-dependent Probe Amplification (MLPA) (MRC-Holland, Amsterdam) was used to detect large exonic *BRCA1* and *BRCA2* rearrangements (only 23 probands were analyzed for the latter gene) in cases that were negative for pathogenic *BRCA1*/*BRCA2* point mutations.

Screening for the c.156_157insAlu *BRCA2* mutation

The c.156_157insAlu *BRCA2* mutation was screened in 208 probands and in 262 control chromosomes (healthy blood donors). In the first PCR we used primers for *BRCA2* exon 3 amplification (forward primer: GTCACTGGT TAAAACTAAGGTGGG and reverse primer: GAAGCCA GCTGATTATAAGATGGTT). The cycling conditions were 94°C for 1 min, 35 cycles of 94°C for 1 min, 52°C for 1 min, and 72°C for 4 min, and a final extension of 72°C for 10 min. In the second PCR we used primers specific for the c.156_157insAlu *BRCA2* mutation (forward primer: GACACCATCCCGGCTGAAA and reverse primer: CCC CAGTCTACCATATTGCAT). The cycling conditions were 97°C for 15 min, 6 cycles of 97°C for 1 min, 68°C for 1 min, and 72°C for 1 min, 6 cycles of 97°C for 1 min, 66°C for 30 s, and 72°C for 1 min, 6 cycles of 97°C for 1 min, 64°C for 30 s, and 72°C for 1 min, 22 cycles of 97°C for 1 min, 60°C for 30 s, and 72°C for 1 min, and a final extension of 72°C for 10 min. Sequence analysis of genomic fragments with the insertion was carried out on an Applied Biosystems' 310 automated DNA sequencer (FosterCity), using the dye terminator method.

RNA isolation and transcript analysis

Frozen cell pellets from nucleated cells of patients carrying the c.155_156insAlu *BRCA2* variant and from control individuals (without known *BRCA1* or *BRCA2* mutations) were used to extract total RNA using 1 ml of Tripure isolation reagent (Roche Diagnostics, Indianapolis, USA), according to the manufacturer's instructions. After quantification using a NanoDrop ND-1000 spetrophotometer, One-Step RT-PCR was performed in all cases using the Superscript One-Step RT-PCR System as suggested by the manufacturer (Invitrogen, Carlsbad, USA). We used primers spanning from exon 1 to exon 6 of *BRCA2* (forward primer B2Ex1F: GCTTACTCCGGCCAAAAAAGA and reverse

primer B2Ex6R: GGTGTCTGACGACCCTTCACA). The cycling conditions were 50°C for 30 min, 95°C for 15 min, 40 cycles of 94°C for 30 s, 55°C for 1 min, and 72°C for 1 min, and a final extension of 72°C for 10 min. RT-PCR products were separated on a 2% agarose gel (SeaKem LE Agarose, Rockland, USA) and visualized with ethidium bromide in an image analyser ImageMaster VDS (Amersham Biosciences, Little Chalfont, UK). Individual bands were excised from the gel, purified using GFX^{TM} PCR DNA and Gel Band Purification Kit (GE HealthCare) and sequenced as indicated above.

Microsatellite typing and haplotype analysis

A total of 14 probands and 14 family members were genotyped for polymorphic microsatellite markers flanking *BRCA2* and for the c.-25G>A polymorphism. The order of the markers, the consensus repeat, and the distances relative to each other and *BRCA2* are shown in Table 1. The physical distances of the genetic markers were derived from the Human Genome Browser assembly hg18 from the UCSC Genome Bioinformatics (http://www.genome.ucsc.edu/). The consensus pattern was obtained with the software Tandem Repeats Finder (http://www.tandem.bu.edu/). The primer sequences for the amplification of the markers were derived from the Human Genome database (http://www.gdb.org). All 10 markers were assayed by PCR using fluorescently end-labeled primers. PCR products were run on an ABI PRISM 310 Genetic Analyser (Applied Biosystems) together with a fluorescence labeled DNA fragment size standard. Genotyping of the c.-25G>A polymorphism was performed by sequencing. Haplotypes were reconstructed manually in the three informative families. The geographic origin of the c.156_157insAlu *BRCA2* positive families was inferred from the birthplace of the oldest carrier or of the oldest affected family member most likely to be a carrier.

Results

Detection of the c.156_157insAlu *BRCA2* mutation

We identified eight probands with the c.156_157insAlu mutation in our initial series of 78 patients in whom no deleterious *BRCA1*/*BRCA2* mutation had been found by DGGE and MLPA. This finding, together with the 22 pathogenic mutations previously reported [14], raised the deleterious mutation detection rate to 30% (30/100) of all cases studied and to 37% (29/79) of those probands with a family history of breast/ovarian cancer. The c.156_157insAlu *BRCA2* mutation accounts for 27% of the 30 probands with *BRCA1*/*BRCA2* pathogenic mutations and

Table 1 Microsatellite marker genotypes used to reconstruct the haplotypes in probands with the c.156_157insAlu *BRCA2* mutation

Markers	Position (Mb)	Consensus pattern	1	2	3	4	5	6	7	8	9	10	11	12	13	14
cen																
D13S1700	0.56	AGAA	257/265	249/257	261/317	309/321	257/309	317/317	257/317	317/325	265/317	313/317	261/317	309/317	253/313	313/317
D13S260	0.45	TG	160/164	160/164	160/160	160/160	162/168	160/168	158/160	168/168	160/160	158/160	160/160	160/166	160/160	160/168
D13S1699	0.28	TTTC	153/153	153/153	153/153	153/153	153/157	153/157	150/153	153/153	150/153	153/153	150/153	153/153	153/157	153/153
D13S1698	0.19	GT	156/156	156/160	156/160	156/156	156/160	156/160	156/160	156/174	156/156	156/156	156/166	156/176	156/160	156/156
c.-25G>A	Intragenic		G/G	G/G	A/G	A/G	A/G	G/G	G/G	G/G	G/G	A/G	G/G	G/G	G/G	G/G
BRCA2																
D13S1701	0.17	TTCC	299/299	295/299	299/299	291/299	295/299	291/299	295/299	295/299	291/299	299/299	295/299	299/307	299/307	291/299
D13S171	0.28	TG	230/230	230/240	230/240	230/230	226/230	226/230	230/230	230/230	230/240	230/230	230/230	226/226	230/240	230/230
D13S1695	0.55	AC	242/250	242/250	242/250	242/246	240/242	242/250	236/246	242/250	242/250	242/258	242/250	250/252	242/250	242/254
D13S1694	0.8	TG	228/234	228/234	228/228	234/236	228/232	228/234	234/234	228/232	234/234	228/234	228/234	234/234	228/234	234/238
D13S310	0.81	GT	144/144	144/144	144/144	148/148	144/144	144/144	148/166	144/148	144/150	144/148	144/144	148/150	144/150	144/148
D13S267	1.29	TG	156/158	144/156	156/158	144/150	156/158	156/158	144/150	144/150	144/144	146/158	150/158	144/158	146/158	144/156
tel																

53% of the 15 deleterious *BRCA2* mutations identified in that series of 100 probands from breast/ovarian cancer families. Furthermore, screening of the c.156_157insAlu *BRCA2* mutation in 130 additional probands in whom no germline *BRCA1*/*BRCA2* mutations have been detected by DGGE and MLPA (data not shown) revealed six additional families with this mutation. Of the 14 probands with the c.156_157insAlu *BRCA2* mutation, eight belonged to ≥2BC families, three to MBC families, two to BC + OC families, and one was a 1BC + OC patient. The mutation was also detected in six affected family members but neither in eight healthy relatives nor in the 262 chromosomes of the control population.

Analysis of RNA transcripts

RNA splicing was evaluated by amplifying the *BRCA2* coding region from exon 1 through exon 6. When the products from these reactions were visualized on agarose gels, besides a normal transcript two shorter bands were common to both the patients and controls: a transcript representing an in frame skip of exon 3 (*BRCA2*-Δex3) and a transcript with deletion of exon 3 plus 109 bp of exon 4 (Fig. 1). However, the band representing the *BRCA2*-Δex3 transcript had much higher intensity in the patients with the c.156_157insAlu *BRCA2* mutation. When these fragments were excised from the gel, amplified, and sequenced, and because the PCR were designed to generate fragments that contained the c.-25G>A polymorphism, we could demonstrate that the controls heterozygous for the c.-25G>A polymorphism showed heterozygosity in the full length and the *BRCA2*-Δex3 transcripts, whereas the patients heterozygous for that polymorphism presented mainly one allele in the full length transcript and the other allele in the *BRCA2*-Δex3 transcript (Fig. 1). These findings indicate that the normal mRNA for *BRCA2* is produced only from one chromosome in the patient.

Haplotype analysis

Haplotypes were phased for most of the markers for only three out of the 14 families. The results of the haplotype analyses for the three informative families are shown in Fig. 2. In one informative family, the haplotype associated with the mutation could be inferred accurately as five carriers were genotyped. In the other two families the haplotype was inferred in most instances as two carriers per family were genotyped. One haplotype was found to segregate within two families (11 and 13). The shared haplotype covered eight markers, but varied for the D13S1701 and D13S1700 alleles. In family 12 the disease associated alleles from the *BRCA2* telomeric region were different from those associated with families 11 and 13. The genotypes found for the remaining probands are shown in Table 1 and the geographic origins of

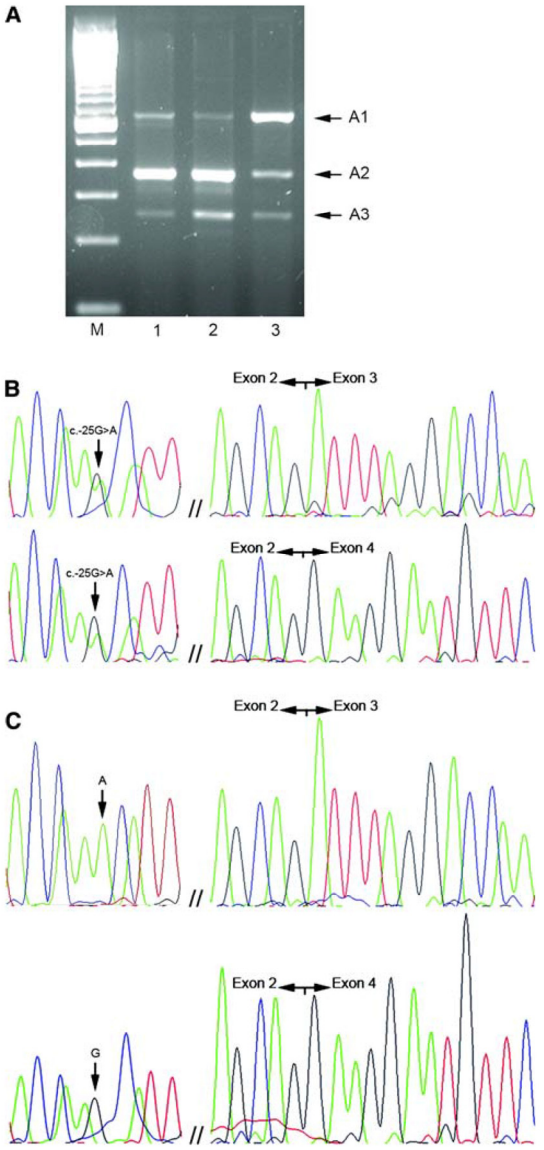

Fig. 1 RT-PCR analyses from exon 1 to exon 6 of the *BRCA2* gene in c.156_157insAlu *BRCA2* mutation carriers and controls. (**a**) Lane 1, 100 bp DNA standard. Lanes 2 and 3, patients with the c.156_157insAlu *BRCA2* mutation. Lane 3, wild type control sample. A1 represents the full length transcript, A2 the *BRCA2*-Δex3 transcript and A3 the transcript with deletion of exon 3 plus 109 bp of exon 4 (data not shown). (**b**) Sequencing of the RT-PCR product (A1 band top and A2 band bottom) from a control individual with the c.-25G>A germline polymorphism (noted by an arrow), showing heterozygosity in the full length and the *BRCA2*-Δex3 transcripts. (**c**) Sequencing of the RT-PCR product (A1 band top and A2 band bottom) from a c.156_157insAlu *BRCA2* mutation carrier with the c.-25G>A germline polymorphism (noted by an arrow), showing mainly one allele in the full length transcript and the other allele in the *BRCA2*-Δex3 transcript

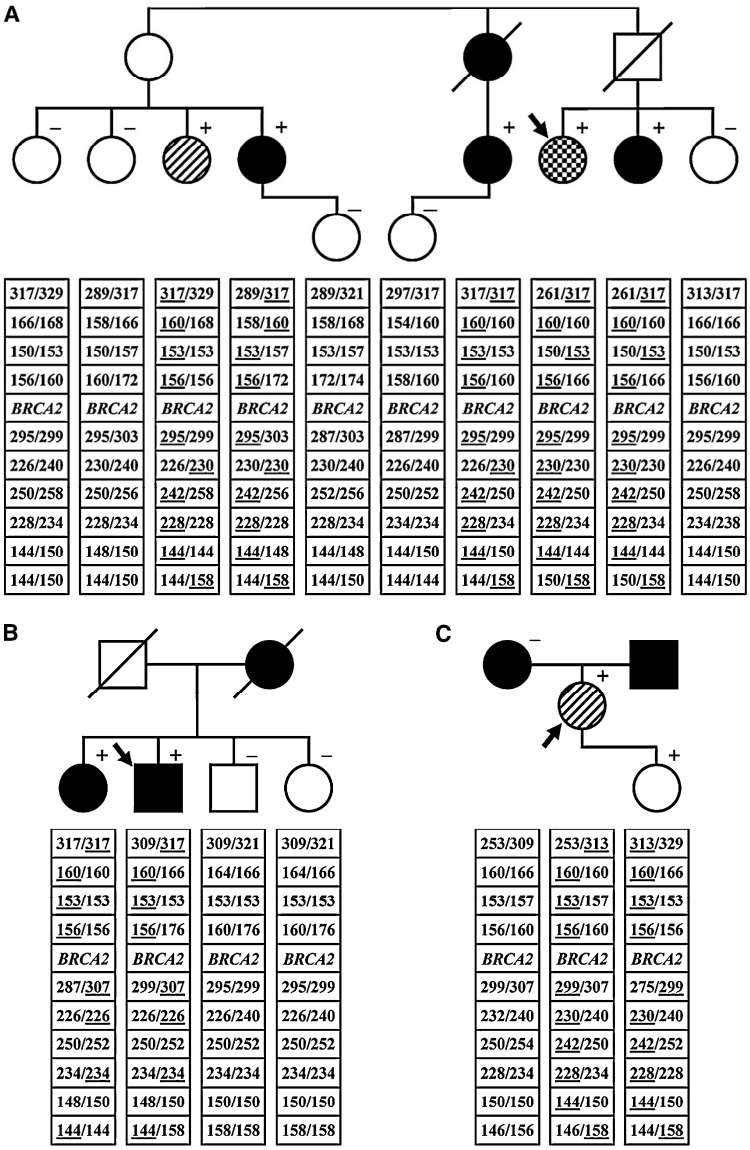

Fig. 2 Pedigrees of the three informative breast/ovarian cancer families and haplotype results (families 11, 12 and 13 in **a**, **b** and **c**, respectively). The order of the microsatellite markers is the same as in Table 1 and the alleles that segregate with the mutation are underlined. Unaffected individuals are indicated with open symbols, patients affected with breast cancer with black symbols. Bilateral breast disease is represented by striped circles and breast/ovarian cancer patients by dotted circles. Plus and minus signals represent family members with and without the c.156_157insAlu *BRCA2* mutation, respectively

the c.156_157insAlu *BRCA2* positive families are shown in Fig. 3.

Discussion

Our findings show that the specific detection of the c.156_157insAlu *BRCA2* mutation in Portuguese breast/ovarian cancer families allows a significant increase in the detection rate of *BRCA1*/*BRCA2* mutations from 24.7% to 37% of those with a family history of cancer. This mutation rate is slightly above what has been reported in the majority of populations using similar criteria for referral to genetic counseling and testing [12, 20–22], something that might reflect the fact that we are now able to detect a mutation that escaped conventional mutation screening techniques. If we had used more strict selection criteria like those of the other major report on *BRCA1*/*BRCA2* analysis of Portuguese breast/ovarian cancer families (BRCAPRO risk >25%, resulting in a mutation detection rate of 36%, one-

Fig. 3 Map of Portugal showing the known geographical origin of the families with the c.156_157insAlu *BRCA2* germline mutation. The open circles indicate the origin of the 14 families here reported and the black circles the families previously described by Machado et al. [16]

sixth being the c.156_157insAlu *BRCA2* mutation [16]), our mutation detection rate would have been 41%. Although this mutation rate can be seen as relatively high, it also indicates that other *BRCA1*/*BRCA2* mutations undetectable by current-day screening methodologies may remain to be uncovered in high-risk breast/ovarian cancer families. On the other hand, five out of the 30 pathogenic mutations would have been missed had we used the generally recommended higher that 10% mutation probability criteria for genetic testing (8 of 30 if >25%). Careful selection of families after genetic counseling is therefore recommended to increase the chance of identifying all families that can benefit from genetic diagnosis of inherited predisposition to breast/ovarian cancer.

Teugels et al. [15] demonstrated that the c.156_157ins Alu mutation originates an in-frame deletion of exon 3 from the *BRCA2* mRNA encoding a transcriptional activation domain. On the other hand, Díez et al. [17] issued a word of caution when interpreting as pathogenic alterations causing *BRCA2* exon 3 skipping, as exon 3 splicing can also be detected in normal tissues. In order to better characterize the pathological and clinical significance of this mutation, we have looked for this mutation in disease-free controls, analyzed in more detail the mRNA in carriers and noncarriers, and reconstructed haplotypes in informative families. Three separate findings corroborate the conclusion that the c.156_157insAlu *BRCA2* mutation is indeed deleterious. First, *BRCA2* full length transcript is derived only from the wild type allele in patients carrying the c.156_157insAlu *BRCA2* mutation, whereas the *BRCA2*-Δex3 transcript was transcribed mostly from the mutated allele. This was demonstrated using a heterozygous polymorphism to evaluate the relative contribution of each allele to normal and *BRCA2*-Δex3 transcripts in patients and controls [23], the latter showing both alleles giving rise to a small proportion of the *BRCA2*-Δex3 transcript. Second, if the abnormal transcript with the in-frame deletion of exon 3 is fully functional, as it was suggested by Díez et al. [17], then this variant would have to be considered a polymorphism given its high frequency (8%) in families suspected to have hereditary predisposition to breast/ovarian cancer. However, we did not find the c.156_157insAlu in 262 chromosomes from healthy blood donors, allowing us to dismiss the hypothesis that it is a neutral polymorphism. Third, we could show co-segregation of this alteration and disease in six affected (but not in healthy) family members belonging to the informative families. These pieces of information allow us to confidently conclude that the c.156_157insAlu *BRCA2* mutation is associated with the hereditary predisposition to breast/ovarian cancer in these families.

Alu elements are believed to insert randomly in the host genome, so the fact that this particular Alu is inserted at exactly the same position in *BRCA2* exon 3 in all 14 families is by itself sufficient evidence that this represents a founder effect and not a recurrent mutation [24]. Additionally, in all c.156_157insAlu mutation carriers heterozygous for the c.-25G>A polymorphism there was a physical association between the mutation and the allele that did not contribute to wild type transcript. Nevertheless, analysis of the chromosomal background upon which a mutation occurs can be used to reconstruct its origin and age. We have performed haplotype reconstruction with ten microsatellite markers in three informative families. In two of the three families one haplotype was shared for all but two markers, whereas in the third family all markers telomeric to *BRCA2* differed from that observed in the other two. A recombination event around *BRCA2* could account for the latter haplotype difference, whereas the variance in allele size of one tetranucleotide repeat unit within the D13S1701 allele in two families may have been

caused by slippage during DNA replication of this repeat sequence. It is not possible to determine if this marker exhibits a higher mutation rate than other markers in the *BRCA2* region, as it was suggested by other studies [25], or if that mutation occurred early on during in the spreading of the population carrying the c.156_157insAlu *BRCA2* mutation. If we compare the D13S1701 genotypes for all 14 c.156_157insAlu positive probands, it is tempting to say that all mutation carriers with unphased genotypes harbored the same 299 allele, but in two out of three informative families we observed that this allele did not segregate with the mutation. The possibility exists that this variation in D13S1701 is more frequent than that observed by Machado et al. [16], so care should be taken when constructing haplotypes from independent families. If that is so, estimation based on the closest recombinant markers could greatly underestimate mutation age. Although Machado et al. [16] claim that the c.156_157insAlu *BRCA2* mutation has it origin 2,400–2,600 years ago in what is now central/southern Portugal, it is likely that it merely reflects their target population for genetic testing. In fact, we here show that the c.156_157insAlu *BRCA2* mutation accounts for 27% of the *BRCA1*/*BRCA2* deleterious mutations in northern/central Portugal (our main target population; Fig. 3), as compared to 16% in central/southern Portugal [16]. Although all known c.156_157insAlu *BRCA2* mutations have so far been identified in Portuguese breast/ovarian cancer families, we cannot rule out that this mutation may be present in other populations and so it might have been introduced in Iberian Peninsula from somewhere else.

In summary, our study provides significantly new evidence to substantiate the pathogenicity of the c.156_157insAlu *BRCA2* rearrangement and shows that this mutation is responsible for more that one-fourth of deleterious *BRCA1*/*BRCA2* mutations in northern/central Portugal. To further evaluate whether or not it constitutes a population-specific founder mutation, one needs to perform the screening of the c.156_157insAlu *BRCA2* rearrangement in other populations, which will allow for a more accurate estimation of its distribution and age.

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PAPER II

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"Molecular diagnosis of the Portuguese founder mutation *BRCA2* c.156_157insAlu"

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LETTER TO THE EDITOR

Molecular diagnosis of the Portuguese founder mutation *BRCA2* c.156_157insAlu

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We wish to reply to the Letter to the Editor written by Machado and Vaz [1] concerning our recently published article on the c.156_157insAlu *BRCA2* founder mutation [2], first discovered by Teugels et al. [3] in a Portuguese patient living in Belgium. That letter has several misstatements about our and others' work that must not remain unchallenged.

Machado and Vaz suggest in their letter [1] that we diagnose the c.156_157insAlu *BRCA2* mutation by subjective evaluation of band intensities after RT-PCR. We think that it is clear in our paper [2], under the subheading "Screening for the c.156_157insAlu *BRCA2* mutation", that we screen for this mutation by using two independent PCRs, one for exon 3 amplification and another specific for the Alu rearrangement. Using this strategy, in positive cases we expect two bands in the first PCR (one band if negative) and one band in the second PCR (none if negative). The second PCR helps to control the first PCR for eventual problems with preferential amplification of the shorter fragment (the normal), whereas the first PCR controls for eventual absence of amplification in the second PCR. This strategy of two independent PCRs (which differs from the nested PCR approach used by Machado et al. [4]), followed by sequencing of the genomic fragments in positive cases, allows the unambiguous detection of the c.156_157insAlu *BRCA2* mutation. In the Results section of our article [2], the molecular diagnosis of families with the c.156_157insAlu *BRCA2* mutation is also described under the separate subheading "Detection of the c.156_157insAlu *BRCA2* mutation", whereas any mention to RT-PCR data appears under the subheading "Analysis of RNA transcripts". Therefore, the reason why we performed RT-PCR with primers located in exons 1 and 6 was not to confirm the mutation, but to deliberately be able to compare the physiologic alternative splice lacking exon 3 in controls with the effect of the exon 3 Alu insertion in carriers. Taking advantage of a known polymorphism in *BRCA2* exon 2 (previously reported as c.-25G>A [2, 5, 6], but described as c.-26G>A according to the recommendations of the Human Genome Variation Society [7], we clearly demonstrated in Fig. 1 of our paper [2] the preferential production of the transcript without exon 3 by the allele with the Alu insertion and its (presumably residual) production by both alleles in non-carriers.

Machado and Vaz [1] state that RT-PCR must be used to confirm the c.156_157insAlu *BRCA2* mutation in each patient and that a reliable confirmation of this rearrangement must be done using the primers in exons 2 and 10 first described by Nordling et al. [8]. Both these claims are unfounded for the following reasons. First, it is not necessary to confirm one given mutation at the RNA level in every new patient once its pathogenic nature is well established, as it is now the case for this *BRCA2* rearrangement. Second, Machado and Vaz claim [1] that these primers amplify specifically the transcript produced from the allele with the Alu insertion, and not the ubiquitous alternative splice transcript, allegedly because only the former transcript includes at least the first part of *BRCA2* exon 10 [1]. They present no data to support this assumption nor do they exist in the literature. In fact, Zou et al. [9] have shown that the full length transcript and the ubiquitous alternative splice differ only by the lack of exon 3 in the latter, so exon 10 does not distinguish them. Additionally, the forward primer described by Nordling

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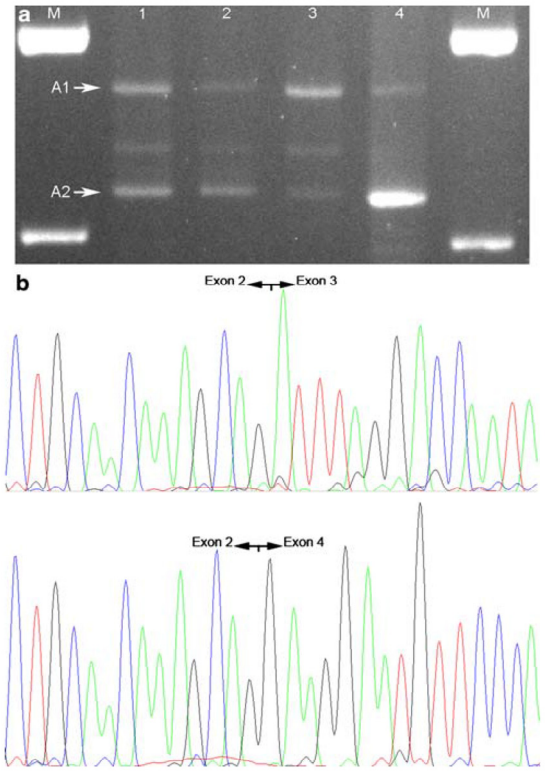

Fig. 1 RT-PCR analyses from exon 1 to exon 10 of the *BRCA2* gene in three normal controls and in one c.156_157insAlu *BRCA2* mutation carrier. **a** *Lanes* M, 1 Kb DNA standard; *Lanes* 1, 2 and 3, controls without the c.156_157insAlu *BRCA2* mutation; *Lane* 4, c.156_157insAlu *BRCA2* mutation carrier; A1 represents the full length transcript and A2 the *BRCA2*-Δex3 transcript. **b** Sequencing of the RT-PCR products (A1 band above and A2 band below) from a control individual showing that both the wild type and the ubiquitous transcript lacking exon 3 can be detected in negative controls

et al. [8] (5′-GCTTATTTACCAAGCATTGGA) has two features that might interfere with the amplification process: first, it contains a mismatch in the first base (A>G); second, it is positioned on the SNP c.-26G>A in exon 2, which has an allelic frequency of 28% [5]. Indeed, Bonnet et al. [10] have recently used a monoallelic RT-PCR protocol based on the SNP c.-26G>A to demonstrate the complete skipping of exon 3 induced by the presence of the c.316+5G>C *BRCA2* mutation. In addition to these methodological flaws, it is likely that Machado et al. [4] were not able to amplify the transcript lacking exon 3 in controls because they try to amplify a large transcript produced at very low level in non carriers, whereas it can more easily be detected in carriers as that transcript is produced at high levels from the mutated allele. To address this possibility, we have analyzed freshly collected blood samples from five negative controls (confirmed not to carry the c.156_157insAlu *BRCA2* mutation). Total RNA was isolated using 1 ml of Tripure isolation reagent (Roche Diagnostics, Indianapolis, USA) and One-Step RT-PCR was performed (QIAGEN GmbH, Germany), as suggested by the manufacturers. Using a 5′UTR *BRCA2* primer that does not include the SNP c.-26G>A (5′-GCTTACTCCGGCCAAAAAAGA) and a reverse primer located in exon 10 (5′-TGGTCACATGAAG AAATATGCAATAG) that allows the detection of its first 469 bp, we were able to amplify both the wild type and the ubiquitous transcript lacking exon 3 in all five negative controls (Fig. 1). These data contradict the assumption made by Machado and Vaz [1, 4] that the alternative splice transcript in negative controls lacks part of exon 10, as we here show using optimized methods for RNA extraction and analysis. We therefore conclude that RT-PCR studies aiming to detect exon 3 skipping should not be performed for molecular diagnosis of the c.156_157insAlu *BRCA2* mutation; instead, this Portuguese founder mutation can reliably be detected by genomic PCR followed by sequencing as we demonstrated in our article [2].

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PAPER III

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

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PRECLINICAL STUDY

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).

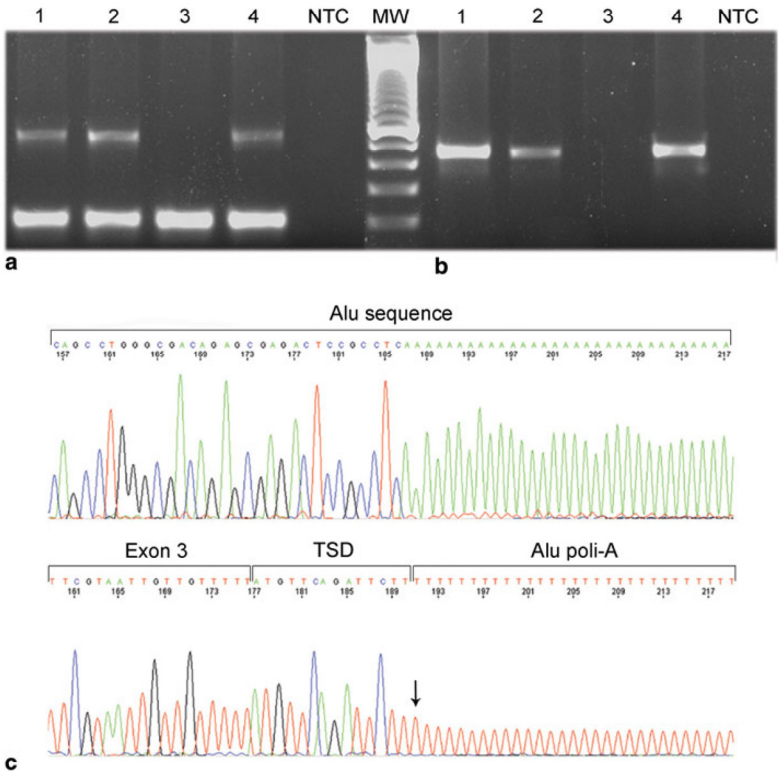

Fig. 1 Molecular diagnosis of the *BRCA2* c.156_157insAlu mutation using two independent PCR analyses, showing positive cases in *lanes 1* and *2* and a negative case in *lane 3*. *Lane 4* corresponds to a positive control and NTC is a non template control. MW refers to 100 bp DNA standard. (**a**) PCR 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]

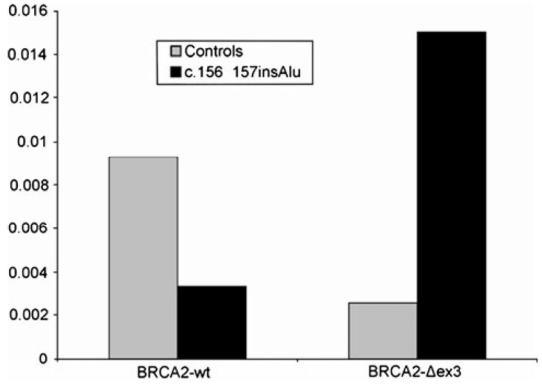

Fig. 2 Real-time RT–PCR quantification of the altered transcript ratios in *BRCA2* c.156_157insAlu carriers as compared with controls. The relative expression of the *BRCA2*-Δex3 transcript was sixfold higher in carriers compared with controls, whereas a threefold decrease was observed for the *BRCA2*-wt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

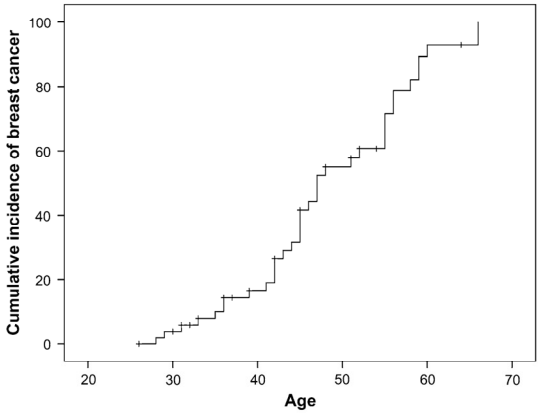

Fig. 3 Cumulative incidence of breast cancer among c.156_157insAlu *BRCA2* germline mutation carriers, reaching 90% at about 60 years of age

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)

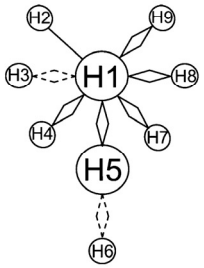

Fig. 4 Phylogenetic network showing the most parsimonious relationships among flanking short tandem repeat-based haplotypes in families carrying the c.156_157insAlu *BRCA2* mutation. *Circle* and *line* sizes are proportional to the number of families and stepwise mutations, respectively. *Diamonds* 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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Material and Method supplementary file

Real-time RT-PCR analysis

The forward primer for the *BRCA2*-wt transcript was TGAAATTTTTAAGACACGCTGCAA and the reverse primer was GGTTCGTAATTGTTGTTTTTATGTTCAG. The forward primer for the *BRCA2*-Δex3 transcript was TCCAAAGAGAGGCCAACATTTT and the reverse primer was TTCACTGTGCGAAGACTTTTATGTC. The probes were 5'-FAM-AAGCAGATTTAGGACCAATA-MGB-3' for the normal transcript (exon 2-3) and 5'-FAM-AACAAAGCAGGAAGGAAT-MGB-3' for the *BRCA2*-Δex3 transcript (exon 2-4). Primers and probes for the *BRCA2* and *GUSB* (endogenous control) genes were purchased from Applied Biosystems. PCR reactions were performed in a 20 µl volume reaction containing 9 µl of synthesized cDNA, 10 µl of TaqMan universal PCR master mix, 1 µM of each primer and 0.25 µM of probe. PCR was performed in separate wells for each primer/probe set and each sample was run in triplicate. PCR parameters were as follows: 50°C for 2 min, 95ºC for 10 min, followed by 50 cycles at 95°C for 15 s and 60°C for 1 min. Each plate included multiple non-template controls and serial dilutions of a control to construct the standard curve of each transcript and endogenous control.

SNaPshot assay primers used for SNP genotyping around *BRCA2*.

	SNP ID	PCR amplification primers	SBE (single base extension) primers	Base change
1	RS379693-F RS379693-R	ACCAGGCACGCATGTAATTC TGCCCAGCCAGAAACTTAAT	ACAGTTGAAAAAGTACTTTCA	A>T
	RS9590624-F RS9590624-R	TTGGGAGGGAGAGCACTAGA TGGGCTCCTACATCCAACTC	**(GACT)$_2$GA**AAGGTTATTGGAGACCTGC	A>T
	RS916732-F RS916732-R	TCACAGAGCTGTGCAAAACC GCGGAATACCATCAACCATC	**(GACT)$_3$**TAGAAAGTAAATGTTTGAAAATT	C>T
	RS2806638-F RS2806638-R	TTCATCCAACCCTTCCAGTC AGCCACAGCAGTGAGAAAAT	**(GACT)$_5$**ATTTTCTTCCTTTCTTTACAC	A>G
	RS206102-F RS206102-R	AAATGAAGCCTCTGTGACAAAA CCAGGGTGTCAGAGGAAAAA	**(GACT)$_7$**GTTTGCATCTTTTTTGAAGG	C>G
2	RS9567445-F RS9567445-R	GCTATGAGGCATAGTCAGCG GCATTTGATGTGGTTAGCATTC	GGGAAACTCAGAAATTCAGT	A>T
	RS798972-F RS798972-R	ACGGCCACCATCTAACAGAC GCAGACGCCTCCTCTCTCTA	**GACT**CAATTTCACCAAAATTTTACCA*	A>T
	RS703219-F RS703219-R	TTAGTGGCCCTGTTCTTGCT AAAAACACCTGGCACAAAGC	**(GACT)$_3$GA**TAGTATTCCATTCCATGTCA*	C>T
	RS582274-F RS582274-R	TCTCACCTGGCTGAAACTCC CTGCTTAGCAACAGGAAGGG	**(GACT)$_4$GA**TTGAAAATAGTTTGATCTCTTT	C>T
	RS176059-F RS176059-R	AAGAAAAAGGGAGAAGTTTGCC TAAACAAATCTGCCCCCATC	**(GACT)$_6$**TTATATTTTGAGTCTAATTTGG	A>G

SNaPshot assay primers used for SNP genotyping around *BRCA2* (continued).

Multiplex	SNP ID	PCR Amplification primers	SBE (single base extension) primers	Base change
3	RS1207952-F RS1207952-R	TGCATGAAGCACACTGTGAA CTCCTTGCTAGCCTCAGGG	GTTCAGCAGTTCTCTTTCAG	A>C
	RS2761367-F RS2761367-R	AGCTGAGGATGGCTGAAAAA GACAAGCAGCCTAAGGGAAG	**GACT**TCATTTTACTTTTTGTTTTGTTT*	G>T
	RS207632-F RS207632-R	GCAACGTTACAGAGCAGTTTG ATGCTCCATCTCCACGTTTC	**(GACT)$_4$**GAGCATCATGTCAATCAATG	C>G
	RS6561643-F RS6561643-F	CCTCAGCAATCCTGTGTGTG GGGACTGGAGGATTTTCTCA	**(GACT)$_5$GA**TGTTAGCTTGTTTTCCAAGT*	A>T
	RS762900-F RS762900-R	GGCAAAAGGCAGTGCTAGTT TCTATACCAATGATGAGCAATCTT	**(GACT)$_6$GAC**TGAATAGGCTTAAAATCTGAA*	A>G
4	RS9595946-F RS9595946-R	TGCCTTTCCCACTCGTTAGT GGCTGAATTGAGTGATGGGT	TCTGAAAAGATTTGTGTTAATA*	A>G
	RS472873-F RS472873-R	TTTCTGCATTGTGACTCTGCTT CACGGTAAAGGTTTCTGGGA	**(GACT)$_2$**AACCAACTTCCTTCGTATTT	T>C
	RS2146994-F RS2146994-R	TCTCACCTGTGGGACCAAAT TAGTGCTGTTCCTTGGGGAC	**(GACT)$_5$GA**TGGAGCTTTGGCCTCTTG	A>C
	RS7983065-F RS7983065-R	TGATGAGACCTGGTAGTCTGTAATG CCCTAGCAATTCTTCTGTATTTG	**(GACT)$_6$**GAAAAAAATGATTTCCCTGAAA	C>T

Supplementary Fig. 1 Map of Portugal showing the known geographical origin of the families with the c.156_157insAlu *BRCA2* germline mutation. The black circles indicate the origin of the 25 families detected in Portugal (present report and those reported in Peixoto et al. [6]), the open circle the origin of the family previously identified by Teugels et al. [4] in Belgium, and the triangles the origin of the families of the four individuals (two from each) subjected to predictive testing living in Rhode Island, USA, and in Villejuif, France, respectively.

H1	**C**	**T**	**A**	**A**	**A**	C/T	**T**	**T**	**G**	**G**		**C**	**T**	**C**	**A**	**G**	**G**	**G**	**C**	**A**
H2	**C**	**T**	**A**	**A**	**A**	**T**	**T**	**T**	**G**	**G**		**C**	**T**	**C**	**A**	**G**	**G**	**G**	**C**	**A**
H3	**C**	**T**	**A**	**A**	**A**	**T**	**T**	**T**	**G**	**G**		**C**	**T**	**C**	**A**	**G**	A/G	A/G	C/T	**A**
H4	C	A	T	**A**	**A**	**T**	**T**	**T**	**G**	**G**		**C**	G/T	**C**	C/A	**G**	**G**	**G**	**C**	**A**
H5	**C**	**T**	**A**	**A**	**A**	**T**	**T**	**T**	**G**	**G**		**C**	**T**	**C**	**A**	**G**	**G**	**G**	**C**	**A**
H6	C/T	A/T	**A**	**A**	**A**	**T**	**T**	**T**	**G**	**G**		**C**	**T**	**C**	**A**	G/C	**G**	**G**	**C**	**A**
H7	**C**	**T**	**A**	**A**	**A**	**T**	**T**	**T**	**G**	**G**		**C**	**T**	**C**	**A**	C	G	A	C	T/A
H8	**C**	**T**	A/T	**A**	A/T	C/T	**T**	**T**	**G**	**G**		**C**	**T**	**C**	**A**	**G**	**G**	**G**	**C**	**A**
H9	C/T	A	**A**	**A**	A/T	**T**	**C**	**T**	A/G	**G**		**C**	**T**	**C**	**A**	G/C	**G**	A/G	**C**	**A**

Supplementary Fig. 2 SNP marker haplotype spanning ~1.1Mb, encompassing the region between the D13S260 and D13S1695 microsatellite markers, in the 11 informative families.

PAPER IV

Ana Peixoto, Manuela Pinheiro, Lígia Massena, Catarina Santos, Pedro Pinto, Patrícia Rocha, Carla Pinto, Manuel R Teixeira

"Genomic characterization of two large Alu-mediated rearrangements of the *BRCA1* gene"

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ORIGINAL ARTICLE

Genomic characterization of two large Alu-mediated rearrangements of the *BRCA1* gene

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To determine whether a large genomic rearrangement is actually novel and to gain insight about the mutational mechanism responsible for its occurrence, molecular characterization with breakpoint identification is mandatory. We here report the characterization of two large deletions involving the *BRCA1* gene. The first rearrangement harbored a 89 664-bp deletion comprising exon 7 of the *BRCA1* gene to exon 11 of the *NBR1* gene (c.441 + 1724_o*NBR1*:c.1073 + 480del). Two highly homologous Alu elements were found in the genomic sequences flanking the deletion breakpoints. Furthermore, a 20-bp overlapping sequence at the breakpoint junction was observed, suggesting that the most likely mechanism for the occurrence of this rearrangement was nonallelic homologous recombination. The second rearrangement fully characterized at the nucleotide level was a *BRCA1* exons 11–15 deletion (c.671-319_4677-578delinsAlu). The case harbored a 23 363-bp deletion with an Alu element inserted at the breakpoints of the deleted region. As the Alu element inserted belongs to a still active AluY family, the observed rearrangement could be due to an insertion-mediated deletion mechanism caused by Alu retrotransposition. To conclude, we describe the breakpoints of two novel large deletions involving the *BRCA1* gene and analysis of their genomic context allowed us to gain insight about the respective mutational mechanism.
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Keywords: Alu retrotransposition; *BRCA1*; homologous recombination; rearrangement

INTRODUCTION

Several hundred distinct *BRCA1* and *BRCA2* mutations have been identified in hereditary breast/ovarian cancer (HBOC) families, including large genomic rearrangements (LGRs). The high content of Alu elements in the intronic sequences of both *BRCA1* and *BRCA2* genes contributes to the occurrence of rearrangements in these genes.[1,2] These genomic rearrangements include primarily deletions and duplications of one or more exons and represent 10–15% of all deleterious germline mutations in the *BRCA1* gene and 1–7% in the *BRCA2* gene.[3,4] The contribution of these LGRs to *BRCA1*/2 mutation-positive families varies in a population-dependent manner and numerous *BRCA1* founder rearrangements have been identified in particular ethnic groups.[5–7] On the other hand, the proportion of rearrangements in the *BRCA2* gene is generally much smaller than that in the *BRCA1* gene, which can be explained by the lower content of repetitive Alu sequences in the former compared with the latter. The only exception to this rule occurs in Portugal, where the founder rearrangement *BRCA2* c.156_157insAlu (an Alu insertion in exon 3[8]) accounts for more than one fourth of HBOC families with deleterious *BRCA1*/*BRCA2* mutations and about half of those with *BRCA2* mutations.[9]

The detection of LGRs requires specific techniques, like multiplex ligation probe amplification (MLPA) or quantitative PCR, which only give information of the exonic region involved in the rearrangement and not on the exact genomic breakpoints. Therefore, rearrangements with distinct breakpoints but involving the same exons may be described similarly, making it difficult to assess whether a given rearrangement is new or even specific of a given population.[10] To determine whether a LGR is actually novel and to gain insight about the mutational mechanism responsible for its occurrence, molecular characterization with breakpoint identification is mandatory. About 81 distinct *BRCA1* LGRs have been described, the majority of them being deletions, followed by rearrangements with both an insertion and a deletion, duplications, one insertion and one triplication.[4] We here report the characterization of the genomic breakpoints of two *BRCA1* LGRs detected in Portuguese HBOC families, namely, a deletion involving *BRCA1* to the nearby gene *NBR1* and an intragenic *BRCA1* deletion.

MATERIALS AND METHODS

Patients and MLPA analyses

Two patients from two HBOC families, initially shown to be negative for *BRCA1* and *BRCA2* point mutations, were included in this study after being shown to carry *BRCA1* exonic rearrangements by MLPA. This study was approved by the Institutional Review Board of the Portuguese Oncology

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Institute-Porto, and written informed consent was obtained from all patients after genetic counseling. DNA was isolated from peripheral blood samples using the salt–chloroform extraction method. Routine screening for *BRCA1* and *BRCA2* exonic rearrangements was performed by MLPA using the P002 kit and the P045 kit, respectively (MRC-Holland, Amsterdam, The Netherlands), according to the manufacturer's instructions. One index patient presenting a deletion from exon 1 to exon 7, c.-232-?_441+?del (NM_007294.3) according to the Human Genome Variation Society nomenclature, had bilateral breast cancer at age 32 and 36, gastric cancer at age 38 and ovarian cancer at age 61, besides having a first-degree relative with breast cancer diagnosed at age 37. The second index patient presented a *BRCA1* deletion including exon 11 to exon 15, described as c.671-?_4675+?del (NM_007294.3) according to the Human Genome Variation Society nomenclature and initially reported in Peixoto *et al.*,[11] had an ovarian borderline tumor at age 38 and breast cancer at age 46, with a first-degree relative presenting breast cancer diagnosed at age 50. These two genomic rearrangements account for two of 48 HBOC families with deleterious *BRCA1* mutations so far identified at the Department of Genetics of the Portuguese Oncology Institute, Porto, Portugal.

Breakpoint identification of the *BRCA1* exons 1–7 deletion

To map the 5′ breakpoint region of this *BRCA1* c.-232-?_441+?del mutation, we used the MLPA kit P239 (MRC-Holland) to evaluate the *BRCA1* promoter region, the *BRCA1* pseudogene (*ΨBRCA1*), and the *NBR2* (exons 1, 3 and 5) and *NBR1* (exons 3, 7 and 11) genes, followed by a semi-quantitative multiplex PCR method spanning intron 11 to exon 22 of the *NBR1* gene (NM_005899.3). Four multiplex reactions were designed, each one containing four controls (exons 10 and 24 of the *BRCA1* gene and exons 11 and 27 of the *BRCA2* gene) and three different *NBR1* regions (Table 1). In addition, all these *NBR1* fragments were designed to include single nucleotide polymorphisms (SNPs) to question, at the same time, their heterozygosity status. The primers for multiplex amplification were designed using the online Primer-BLAST tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). AutoDimer (http://www.cstl.nist.gov/strbase/NIJ/AutoDimer.htm) was used to test for potential hairpin structures and primer dimers. Subsequently, primers were designed spanning the putative breakpoints and PCR was carried out using standard conditions. PCR fragments containing the suspected weight were sequenced with BigDye Terminator cycle sequencing chemistry on an ABI PRISM 310 automatic sequencer (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's recommendations.

Breakpoint identification of the *BRCA1* exons 11–15 deletion

The strategy for breakpoint identification of the *BRCA1* c.671-?_4675+?del mutation was based on the heterozygosity status of a set of SNP markers localized in introns 10 and 15 of *BRCA1*. The selected SNPs were rs7503154, rs8176214, rs77152443 and rs72130855. Subsequently, primers were designed flanking the putative breakpoints and PCR was carried out using standard conditions. PCR fragments were sequenced with BigDye Terminator cycle sequencing chemistry on an ABI PRISM 310 automatic sequencer (Applied Biosystems), according to the manufacturer's recommendations.

Breakpoint sequence context analysis

Breakpoints were defined as a set of coordinates in the genome spanning the genomic sequence of the deletion. Bioinformatic analyses were carried out to assess the genomic context of the region. Both Censor Server (http://www.girinst.org/)[12] and the RepeatMasker software (http://www.repeatmasker.org) were used to search for low-complexity DNA sequences and interspersed repeats in both breakpoint regions. All sequence alignments were performed using the complementary strands to match the Alu consensus sequence.[13]

RESULTS

Characterization of the *BRCA1* exons 1–7 deletion

Analysis by the MLPA kit P002 revealed a genomic deletion encompassing *BRCA1* exons 1–7. Analysis with the MLPA kit P239 of the *BRCA1* promoter region, *ΨBRCA1* and the *NBR2* (exons 1, 3 and 5) and *NBR1* (exon 3, 7 and 11) genes revealed that all these regions were also deleted. To analyze the remaining *NBR1* exons, we performed a semi-quantitative multiplex PCR method spanning intron 11 to exon 22 of the *NBR1* gene. All these *NBR1* fragments included SNPs to question, at the same time, their heterozygosity status (Table 1). The results showed that the *NBR1* region encompassing rs112693602 (intron 11/ exon 12) to rs13119 (exon 22) was not deleted, narrowing the distance to the breakpoint. After long-range PCR with putative primers spanning intron 7 of the *BRCA1* gene and intron 11 of the *NBR1* gene, we obtained an ~1300-bp fragment in the sample with the *BRCA1* rearrangement. Sequence analysis of this PCR product revealed that the case harbored a 89 664-bp deletion, comprising exon 7 of the *BRCA1* gene to exon 11 of the *NBR1* gene, with the 5′ and 3′ breakpoints located 1724-bp upstream of *BRCA1* exon 7 and 480-bp upstream of *NBR1* exon 11, respectively, corresponding to the mutation *BRCA1* c.441+1724_o*NBR1*:c.1073+480del (Figure 1). The 5′ and 3′ breakpoint flanking regions presented a complete homology sequence of 20 bp. Two Alu elements were found in the genomic sequences flanking the deletion breakpoints in *BRCA1* intron 7 (AluSc) and *NBR1* intron 11 (AluSg), and sequence alignment indicated that these two elements were highly homologous (Figure 2a).

Table 1 *NBR1* gene analysis using SNPs and a semi-quantitative multiplex PCR method to characterize the *BRCA1* exons 1–7 deletion

Multiplex	*SNP ID*	NBR1 *gene location*	*Semi-quantitative region information*	*Heterozygosity status*
1	rs35995789	Exon/intron 18	Not deleted	Heterozygous
	rs3744243	Intron 20	Not deleted	Not informative
	rs71379212	Intron 21	Not deleted	Heterozygous
2	rs13119	Exon 22	Not deleted	Heterozygous
	rs35166890	Intron 15	Not deleted	Heterozygous
	rs34743495	Exon/intron 17	Not deleted	Heterozygous
3	rs111808644	Intron 14/exon 15	Not deleted	Not informative
	rs17527933	Exon/intron 20	Not deleted	Heterozygous
	rs112693602	Intron 11/exon12	Not deleted	Not informative
4	rs8482	Exon 22	Not deleted	Heterozygous
	rs111691171	Intron 21	Not deleted	Not informative
	rs2306829	Exon/intron 19	Not deleted	Heterozygous

Characterization of the *BRCA1* exons 11–15 deletion

The breakpoint identification of the *BRCA1* exons 11–15 deletion was based on the information obtained through the study of SNPs located in introns 10 (rs7503154) and 15 (rs8176214, rs77152443 and rs72130855) of the *BRCA1* gene. Markers rs7503154 and rs72130855 were heterozygous, indicating that these regions were not deleted. Markers rs8176214 and rs77152443 were not heterozygous, possibly suggesting that they were located within the deleted region. After long-range PCR with the forward primer of marker rs7503154 and the reverse primer of marker rs72130855, we obtained a fragment of ~1100-bp in the sample with the *BRCA1* rearrangement. Sequence analysis of this PCR product revealed the breakpoint region in the mutated allele. The case harbored a 23 363-bp deletion, comprising exons 11–15 of the *BRCA1* gene with the 5′ and 3′ breakpoints located 319-bp upstream of exon 11 and 578-bp upstream of exon 16 (Figure 1). In addition, at the deletion breakpoint, a sequence of ~250-bp was inserted (Figure 1). Although

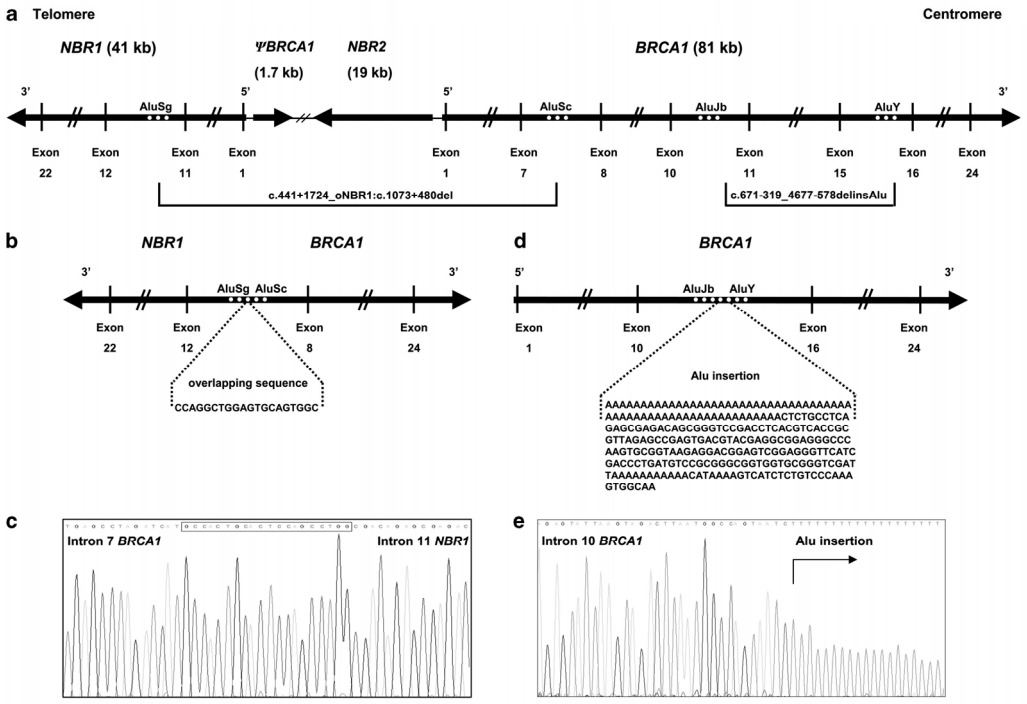

Figure 1 Scheme representing the molecular characterization of the *BRCA1* c.441+1724_oNBR1:c.1073+480del and c.671-319_4677-578delinsAlu mutations. (**a**) Genomic region encompassing the *NBR1* and *BRCA1* genes, with the deleted regions and flanking Alu elements represented for both mutations. (**b**) *BRCA1* c.441+1724_oNBR1:c.1073+480del mutation with the 5′ and 3′ breakpoints located in intron 7 and 11 of *BRCA1* and *NBR1*, respectively. The deletion breakpoints are flanked by two AluSc/AluSg elements with a complete homology sequence of 20 bp within these Alu repeats. (**c**) Sequence electrophorogram of the *BRCA1* c.441+1724_oNBR1:c.1073+480del breakpoint with the overlapping sequence highlighted. (**d**) *BRCA1* c.671-319_4677-578delinsAlu mutation, with the breakpoints in introns 10 and 15 of the *BRCA1* gene and presenting an Alu insertion. The 5′ breakpoint of the deletion was near an AluJb element, whereas the 3′ breakpoint occurred in an AluY element. (**e**) Sequence electrophorogram of the 5′ breakpoint region of the *BRCA1* c.671-319_4677-578delinsAlu, showing a stretch of the poly-A inserted Alu element. A full color version of this figure is available at the *Journal of Human Genetics* journal online.

the exact length could not be assigned because of the inherent difficulties in sequencing long poly-A tracts, this fragment contained ~60-bp poly-A. The Censor Server and RepeatMasker software showed that this sequence was an Alu element belonging to the AluY family, which allowed us to describe this rearrangement as *BRCA1* c.671-319_4677-578delinsAlu. The 5′ breakpoint of the deletion was 120-bp downstream from an Alu element (AluJb), whereas the 3′ breakpoint occurred in another Alu element (AluY). Sequence alignment of the three Alu elements supposedly involved in this rearrangement is represented in Figure 2b. The inserted Alu sequence presented a higher homology to the intron 15 breakpoint region of the *BRCA1* gene.

DISCUSSION

According to Sluiter and van Rensburg,[4] 81 and 17 different LGRs have been characterized for the *BRCA1* and *BRCA2* genes, respectively. One of the mechanisms causing major gene rearrangements in humans involves Alu elements, which are retrotransposable interspersed repetitive sequences that use the L1-encoded transposition machinery and constitute ~10% of the human genome.[14] Alu sequences comprise three major subfamilies, namely, the J (oldest) and S (intermediate) subfamilies that are responsible for the large majority of Alu copies currently present in the human genome, and the Y subfamily (youngest) that is responsible for all of the recently integrated Alu sequences in the human genome.[15,16] These repetitive sequences are responsible for creating genetic variation and also human diseases, not only because Alu sequences are known to mediate the occurrence of genetic recombination events as a direct result of their abundance and sequence homology, but also due to the occurrence of insertion mutations as a consequence of Alu retransposition events.[17]

We characterized two large deletions involving the *BRCA1* gene, which account for about 4% (2/48) of all *BRCA1* HBOC families identified in our population. The first rearrangement we here report harbored a deletion comprising exon 7 of the *BRCA1* gene to exon 11 of the *NBR1* gene (c.441+1724_o*NBR1*:c.1073+480del). Two highly homologous Alu elements were found in the genomic sequences flanking the deletion breakpoint, namely, an AluSc in *BRCA1* intron 7 and an AluSg in *NBR1* intron 11. The alignment of these intronic sequences suggested that the most likely mechanism for the occurrence of this rearrangement was nonallelic homologous recombination between these Alu repeats, substantiated by the presence of a 20-

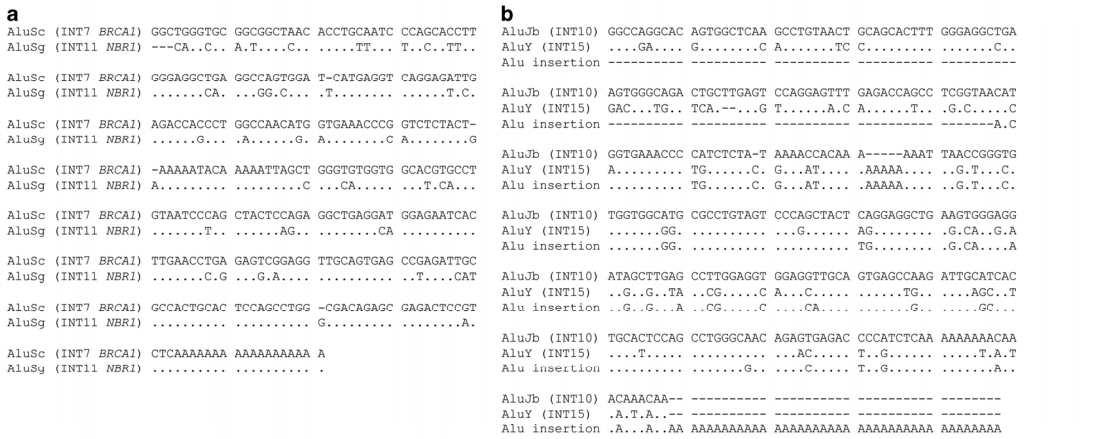

Figure 2 Alignment of the Alu elements in the BRCA1 rearrangements. (**a**) Alignment of the Alu elements involved in the *BRCA1* c.441+1724_oNBR1:c.1073+480del rearrangement, namely, an AluSc in *BRCA1* intron 7 (INT7) and an AluSg *in NBR1* intron 11 (INT11). (**b**) Alignment of the Alu elements involved in the *BRCA1* c.671-319_4677-578delinsAlu rearrangement, an AluJb in *BRCA1* intron 10 (INT10), an AluY in intron 15 (INT15) and the Alu element inserted (Alu insertion). Each dot stands for the same nucleotide and dashes indicate gaps introduced so as to maximize alignment.

bp overlapping sequence at the breakpoint junction. We hypothesize that this sequence contains a recombinogenic hotspot as assumed for other sequences in Alu elements, as also postulated before.[18,19] Rüdiger *et al.*,[19] suggested that four different rearrangements in the low density lipoprotein receptor gene presented a 26-bp common sequence that functions as a recombinational hotspot. This core sequence contains a pentanucleotide motif (CCAGC), which is also part of *chi*, an 8-bp sequence known to stimulate *recBC*-mediated recombination in *Escherichia coli*.[19] We compared our 20-bp overlapping sequence with the sequences involved in the 81 *BRCA1* rearrangements described by Sluiter and van Rensburg.[4] This 20-bp sequence was observed (up to a maximum of three mismatches without affecting the pentanucleotide motif) at the breakpoint region in four rearrangements (NG_005905.2: g.114552_121641del, g.148011_151126del, g.153110_161767dup and g.166375_170153delins), supporting the possibility that these rearrangements were induced by a specific sequence with a recombinogenic nature (Figure 3).[4,19] However, this 20-bp sequence is highly conserved as it is present in the consensus sequence of the three major Alu subfamilies (J, S and Y), which alone can explain the high incidence of homologous recombination involving these sequences.[15] A deletion of exons 1–7 of the *BRCA1* gene was previously described by Engert *et al.*,[20] but the genomic breakpoint was not determined. However, they confirmed by comparative genomic hybridization that the minimum size of the deletion was about 200 kb and the maximum size was about 285 kb encompassing the entire *NBR1* region, so one can assume that the *BRCA1* c.441+1724_o*NBR1*:c.1073+480del we here report is a novel genomic rearrangement. This *BRCA1* LGR encompasses three more *loci*, namely, *NBR1*, *ΨBRCA1* and *NBR2*. No recognizable biological function has been assigned to *ΨBRCA1* and *NBR2*,[21] whereas the *NBR1* gene encodes a highly conserved multidomain scaffold protein involved in selective autophagy.[22] The family here reported does not show any remarkable phenotypic trait apart from being predisposed to breast and ovarian cancers, indicating that the relevant genetic defect is the inactivation of the *BRCA1* gene. A contiguous gene deletion syndrome was also excluded by Gad *et al.*,[23] in one family presenting a *BRCA1* deletion of exons 1–22 that also encompassed the *NBR1*, *ΨBRCA1* and *NBR2* genes. The absence of mutations in the *NBR1* and *NBR2* genes in breast and ovarian cancer patients also indicates that *BRCA1* inactivation is the relevant consequence of this LGR.[24,25]

The second rearrangement fully characterized at the nucleotide level was the *BRCA1* mutation c.671-319_4677-578delinsAlu, previously described as EX11_15del by our group.[11] The 3′ breakpoint occurred in an AluY element and the 5′ breakpoint was 120 bp downstream from a second Alu element (AluJb) in the *BRCA1* gene. In addition, an ~250-bp Alu fragment was inserted at the breakpoints of the deleted region. This Alu fragment presents a high homology to the intron 15 breakpoint region of the *BRCA1* gene and, according to the Censor Server and RepeatMasker software, this sequence is an Alu element belonging to the AluY family. Alu-mediated unequal homologous recombination is the most common mechanism causing *BRCA1* LGRs. The deletion/insertion event here described could be due to homologous recombination with exchange of genetic material.[26] However, another possible explanation for the observed rearrangement could involve an insertion-mediated deletion mechanism caused by the retrotransposition of Alu elements at this genomic site leading to the deletion of adjacent genomic regions.[26–29] This hypothesis is corroborated by the fact that the inserted Alu element belongs to a still active AluY family. There are only a few reports of large deletions caused by the retrotransposition of an Alu element: for instance, a complex deletion–insertion rearrangement has been reported in a patient with lipoprotein lipase deficiency disorder caused by an Alu retrotransposition-mediated mechanism[29] and the *TP53* gene has been shown to be rearranged by a complex rearrangement resulting in deletion of exons 2–4 with an insertion of a truncated Alu fragment.[26] Only eight different deletion–insertion rearrangements have been reported in *BRCA1*, but none attributable to an Alu-mediated retrotransposition mechanism,[4] so this would be

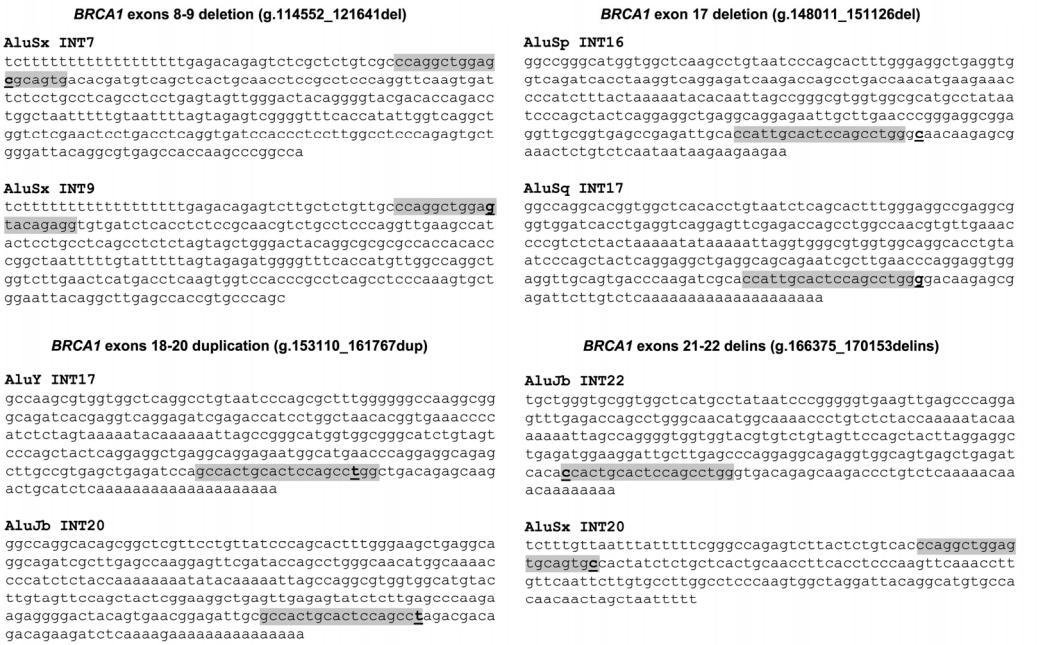

Figure 3 Flanking sequences of four different *BRCA1* gene rearrangements presenting the 20-bp overlapping sequence (gray colored boxes) near or at the breakpoint region, which have been previously reported. The genomic breakpoints (underlined) were obtained from Sluiter and van Rensburg using the RefSeqGene record, NG_005905.2.[4]

the first description of such a mechanism of mutation of the *BRCA1* gene. The deletion of exons 11–15 of the *BRCA1* gene was also described by de la Hoya *et al.*;[30] however, the mutational mechanism and the genomic breakpoint (L78833:g.33450_56562del) are different from those we here report, emphasizing the importance of breakpoint characterization to determine whether rearrangements involving the same exons are related or have occurred *de novo*.

In conclusion, we have identified the breakpoints of two novel large deletions involving the *BRCA1* gene, one encompassing exons 1–7 in addition to the two adjacent genes *NBR2* and *NBR1* (c.441 + 1724_o*NBR1*:c.1073 + 480del) and another comprising exons 11–15 (c.671-319_4677-578delinsAlu). Both rearrangements most likely were mediated by Alu elements, either by homologous recombination between Alu repeats or by the retrotransposition of an Alu element.

ACKNOWLEDGEMENTS

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PAPER V

Ana Peixoto, Catarina Santos, Pedro Pinto, Manuela Pinheiro, Patrícia Rocha, Carla Pinto, Susana Bizarro, Isabel Veiga, Sofia A. Príncipe, Sofia Maia, Fernando Castro, Rosário Couto, Alfredo Gouveia, Manuel R. Teixeira

"The role of targeted *BRCA1*/*BRCA2* mutation analysis in hereditary breast/ovarian cancer families of Portuguese ancestry"

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Short Report

The role of targeted *BRCA1/BRCA2* mutation analysis in hereditary breast/ovarian cancer families of Portuguese ancestry

Peixoto A, Santos C, Pinto P, Pinheiro M, Rocha P, Pinto C, Bizarro S, Veiga I, Principe AS, Maia S, Castro F, Couto R, Gouveia A, Teixeira MR. The role of targeted *BRCA1/BRCA2* mutation analysis in hereditary breast/ovarian cancer families of Portuguese ancestry.
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We report the analysis of altogether 1050 suspected hereditary breast/ovarian cancer (HBOC) families, 524 fully screened for *BRCA1/BRCA2* mutations and 526 tested only for the most common mutations. Of the 119 families with pathogenic mutations, 40 (33.6%) had the *BRCA2* c.156_157insAlu rearrangement and 15 (12.6%) the *BRCA1* c.3331_3334del mutation, the former being specific of Portuguese ancestry and the latter showing a founder effect in Portugal. Interestingly, the two most common mutations were found in a significant proportion of the HBOC families with an *a priori* BRCAPRO mutation probability <10%. We recommend that all suspected HBOC families from Portugal or with Portuguese ancestry, even those fulfilling moderately stringent clinical-criteria for genetic testing, should be specifically analyzed for the two most common *BRCA1/BRCA2* founder mutations, and we here present a simple method for this first tier test. Screening of the entire coding regions of *BRCA1* and *BRCA2* should subsequently be offered to those families with a mutation probability ≥10% if none of those founder mutations are found.

Conflict of interest

The authors declare no conflict of interest.

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Key words: *BRCA1/BRCA2* genes – founder mutations – genetic testing criteria and strategy – Portuguese ancestry

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The pattern of *BRCA1/BRCA2* mutations in hereditary breast/ovarian cancer (HBOC) families varies widely among different populations, some of which show a high frequency of unique mutations. This is the case of the c.156_157insAlu *BRCA2* rearrangement, which has been identified only in HBOC families from Portugal or of Portuguese ancestry (1–3). As this rearrangement is not readily detectable by regular or next-generation mutation screening (1, 3, 4) and there are millions of Portuguese emigrants and individuals with Portuguese ancestry with a worldwide distribution (www.observatorioemigracao.pt), it is very important to call attention to the necessity of specifically testing for this mutation in such cases.

Identification of frequent (recurrent and/or founder) mutations is an extremely important step to increase the efficiency of genetic testing, as it makes possible the use of more specific, cheaper and quicker strategies to identify HBOC families (5). We have previously recommended that all suspected HBOC families from Portugal or with Portuguese ancestry should be specifically tested for the *BRCA2* c.156_157insAlu rearrangement, ideally prior to screening the entire coding regions of *BRCA1* and *BRCA2* (2). On the other hand, the existence

of founder mutations may also allow widening the criteria for genetic testing, potentially enabling the diagnosis of HBOC patients with an *a priori* BRCAPRO mutation probability under the commonly used testing threshold of 10%. However, the criteria for genetic testing of Portuguese founder mutations have not been established.

In this study, we report the analysis of altogether 1050 suspected HBOC families in order to characterize in more detail the mutation pattern of the *BRCA1*/*BRCA2* genes, to evaluate the impact of the existence of founder mutations for genetic testing criteria, and to determine the best strategy for molecular testing of HBOC families of Portuguese ancestry.

Materials and methods

Material

This study includes 524 suspected HBOC families that were selected for *BRCA1* and *BRCA2* full mutation screening based on clinical-criteria (6) or, more recently, on the *a priori* probability of finding a mutation using the BRCAPRO software (≥10%). Additionally, 526 suspected HBOC families with a BRCAPRO mutation probability <10% were selected for screening of the most frequent mutations in each gene, based on the mutation frequencies observed in the 524 families in which full screening of *BRCA1* and *BRCA2* was performed. BRCAPRO mutation probability was also retrospectively calculated for the group of probands that had been selected on the basis of clinical-criteria. Samples for genetic testing and clinicopathological data were obtained after genetic counseling according to institutional review board approved guidelines.

BRCA1/*BRCA2* mutation screening

Mutation screening of the entire coding regions of *BRCA1* and *BRCA2* was performed as previously described (1). For the detection of the c.156_157insAlu rearrangement we established a semiquantitative multiplex polymerase chain reaction (PCR) method, which was initially validated using samples with that rearrangement. Briefly, short fragments (reference DNA regions) were coamplified with exon 3 (target sequence) of the *BRCA2* gene by PCR using fluorescently end-labeled primers. The c.3331_3334del mutation located in *BRCA1* exon 11 was screened using fluorescently end-labeled primers specific for that region. The PCR products obtained from the semiquantitative multiplex PCR and from the c.3331_3334del mutation PCR reaction were, for each sample, mixed and run on an ABI PRISM 310 Genetic Analyser (Fig. 1). PCR cycling conditions and primers are available on request.

Haplotype analysis

A total of 15 probands and 51 family members with the c.3331_3334del and seven probands and 29 family members with the c.2037delinsCC *BRCA1* mutations were genotyped for polymorphic microsatellite markers flanking *BRCA1* (Table S1, Supporting information). Haplotypes were reconstructed manually in the informative families.

Statistical analysis

Fisher's exact test was used to compare the clinicopathological data of *BRCA1*/*BRCA2* breast tumors and the frequencies of *BRCA1*/*BRCA2* founder mutations between the two groups with BRCAPRO mutation probability <10%. Statistical significance was considered whenever $p < 0.05$.

Results

BRCA1 and *BRCA2* mutation screening

Of the 524 probands screened for germline *BRCA1* and *BRCA2* mutations, 112 (21.4%) harbored deleterious mutations. Additionally, seven (1.3%) mutations were detected in the 526 families with a BRCAPRO mutation probability <10%, three in the *BRCA1* gene (c.3331_3334del) and four in the *BRCA2* gene (c.156_157insAlu). The spectrum of the *BRCA1* and *BRCA2* mutations is summarized in Table 1. In total, 119 probands with pathogenic mutations were detected, 49 (41.2%) in *BRCA1* and 70 (58.8%) in *BRCA2*, comprising 24 different *BRCA1* mutations and 22 different *BRCA2* mutations. Of the 119 families with pathogenic mutations, 33.6% (40/119) had the *BRCA2* c.156_157insAlu rearrangement, 12.6% (15/119) the *BRCA1* c.3331_3334del mutation, 5.9% (7/119) the *BRCA1* c.2037delinsCC mutation, and 4.2% (5/119) the *BRCA2* c.9097dup mutation. Less frequent mutations were present in the remaining 43.7% (52/119) of the families.

Variants of unknown significance (VUS) were identified in 75 (14.3%) of 524 families in which mutation screening of the entire *BRCA1*/*BRCA2* coding regions was performed. Seventy different VUS (26 and 44 different variants in *BRCA1* and *BRCA2* genes, respectively) were identified, of which seven were detected in probands with *BRCA1*/*BRCA2* pathogenic mutations (Table 2). Nineteen variants were previously classified as either neutral or polymorphisms (7) and were therefore not included here.

BRCAPRO mutation probabilities

BRCAPRO mutation probability was retrospectively calculated in the group of probands previously selected on the basis of clinical-criteria (in eight patients probabilities could not be estimated because of lack of detailed family history information). Of the total 524 families fully screened based on clinical-criteria, 201 presented BRCAPRO mutation probabilities <10% and 19 of these were carriers of a pathogenic mutation, seven of them with the c.156_157insAlu, one with the c.3331_3334del, and 11 with other deleterious mutations in either gene. When the two groups with BRCAPRO mutation probabilities

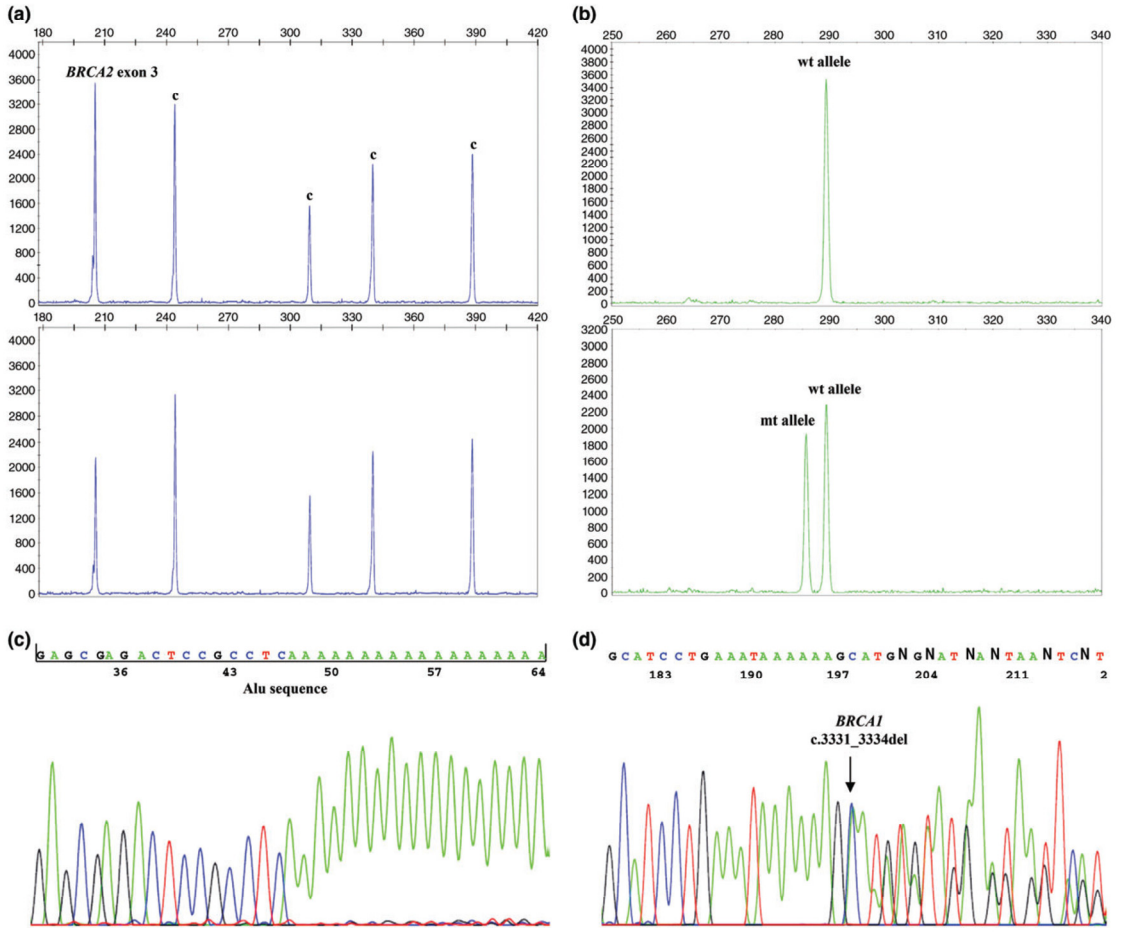

Fig. 1. (**a**) Detection of the *BRCA2* c.156_157insAlu rearrangement using a semiquantitative multiplex polymerase chain reaction (PCR) method. Capillary electrophoresis pattern from a *BRCA2* c.156_157insAlu carrier (lower panel) presenting a signal reduction of approximately 50% of *BRCA2* exon 3 compared with a normal control sample (upper panel). Reference DNA regions that are used for normalization are indicated by c. (**b**) Detection of the *BRCA1* c.3331_3334del mutation by fragment analysis. Capillary electrophoresis pattern from a *BRCA1* c.3331_3334del carrier (lower panel) and a non-carrier (upper panel) showing two peaks (wild-type and mutant allele with 4 bp deletion) and one peak (wild-type alleles), respectively. (**c**) Sequence electropherogram of the amplified genomic fragment of a *BRCA2* c.156_157insAlu carrier. (**d**) Sequence electropherogram of the amplified genomic fragment of a *BRCA1* c.3331_3334del carrier (arrow).

<10% were compared, the estimated probability of having a pathogenic mutation in the group of 201 families fully screened was higher (median 0.039, range 0.001–0.095) than in those selected for screening of frequent mutations (median 0.023, range 0.000–0.093) ($p < 0.0001$).

Haplotype analysis

Regarding the c.2037delinsCC *BRCA1* mutation, a haplotype was phased for two of seven families. The remaining five families presented a haplotype compatible with the one phased for markers D17S855, D17S1323, and D17S1327.

For the *BRCA1* c.3331_3334del mutation, a haplotype was phased in six of 15 families. The other families carrying this mutation harbored a haplotype compatible with the one phased for all the markers, with the exception of the D17S579 marker (Table S1).

Clinicopathological analysis

Histopathological analysis of breast ($n = 80$) and ovarian carcinomas ($n = 17$) from probands with deleterious mutations is summarized in Table S2.

Discussion

We have here characterized the mutational spectrum of germline *BRCA1/BRCA2* mutations in 1050 Portuguese breast/ovarian cancer families, of which 524 were fully screened. Inherited cancer predisposition could be linked

Table 1. Pathogenic *BRCA1/BRCA2* mutations in Portuguese breast/ovarian cancer families

Exon/intron	Base change[a]	Aminoacid change	Reported in BIC[b]	*N* families
BRCA1				
2	c.66dup	p.(Glu23ArgfsTer18)	Yes	1
5	c.181T>G	p.(Cys61Gly)	Yes	1
5	c.211A>G		Yes	3
8	c.470_471del	p.(Ser157Ter)	Yes	2
11	c.1969C>T	p.(Gln657Ter)	No	1
11	c.2037delinsCC	p.(Lys679AsnfsTer4)	No	7
11	c.2086dup	p.(Thr696AsnfsTer16)	No	1
11	c.2418del	p.(Ala807HisfsTer8)	No	1
11	c.2490_2497dup	p.(Leu833CysfsTer16)	No	1
11	c.2906del	p.(Asn969IlefsTer31)	No	1
11	c.3257T>A	p.(Leu1086Ter)	Yes	1
11	c.3331_3334del	p.(Gln1111AsnfsTer5)	Yes	15
11	c.3481_3491del	p.(Glu1161PhefsTer3)	Yes	1
11	c.3627dup	p.(Glu1210ArgfsTer9)	Yes	1
11	c.3817C>T	p.(Gln1273Ter)	Yes	2
11	c.4035del	p.(Glu1346LysfsTer20)	Yes	1
12	c.4136_4137del	p.(Ser1379Ter)	No	1
14	c.4484G>T		Yes	1
16	c.4891del	p.(Ser1631ValfsTer2)	No	1
17	c.5030_5033del	p.(Thr1677IlefsTer2)	Yes	1
20	c.5266dup	p.(Gln1756ProfsTer74)	Yes	2
Intron 20	c.5278-1G>T		Yes	1
11–15	c.671-319_4677-578delinsAlu	p.?	No	1
1–7	c.441+1724_oNBR1:c.1073+480del	p.?	No	1
BRCA2				
3	c.156_157insAlu[c]		No	40
Intron 8	c.682-2A>C		No	2
10	c.956del	p.(Asn319IlefsTer5)	No	1
10	c.1368_1369dup	p.(Lys457ArgfsTer4)	No	1
11	c.2808_2811del	p.(Ala938ProfsTer21)	Yes	1
11	c.3680_3681del	p.(Leu1227GlnfsTer5)	Yes	1
11	c.4380_4381del	p.(Ser1461LeufsTer4)	No	1
11	c.4808del	p.(Asn1603ThrfsTer14)	No	1
11	c.4964dup	p.(Tyr1655Ter)	No	2
11	c.5355dup	p.(Ser1786Ter)	No	1
11	c.5645C>A	p.(Ser1882Ter)	Yes	2
11	c.5653dup	p.(Cys1885LeufsTer15)	No	1
11	c.5722_5723del	p.(Leu1908ArgfsTer2)	Yes	1
13	c.7007G>A		Yes	1
16	c.7631del	p.(Gly2544AlafsTer7)	No	1
Intron 17	c.7977-1G>A		No	1
Intron 19	c.8488-1G>A		No	2
21	c.8695C>T	p.(Gln2899Ter)	No	1
Intron 22	c.8954-5A>G		No	1
23	c.9097dup	p.(Thr3033AsnfsTer11)	No	5
23	c.9097del	p.(Thr3033LeufsTer29)	No	1
25	c.9382C>T	p.(Arg3128Ter)	Yes	2
Total				70

[a]The nomenclature of mutations is in accordance to the Human Genome Variation Society (HGVS) recommendations (version 2.121101). The DNA numbering is based on the cDNA sequences, NM_007294.3 for *BRCA1* and NM_000059.3 for *BRCA2*.
[b]BIC: Breast Cancer Information Core database (http://research.nhgri.nih.gov/bic/).
[c]This mutation is not described according to the HGVS nomenclature, as the inserted sequence is flanked by a short sequence duplication (TSD) (3). See Fig. S1 for detailed information regarding the inserted Alu element.

Table 2. *BRCA1/BRCA2* variants of unknown significance in Portuguese breast/ovarian cancer families

Exon/intron	Base change[a]	Aminoacid change	BIC[b] (clinically important[c])	Additional data
BRCA1				
1–3	c.-232-?_134+?dup		Not reported	
Intron 2	c.80+5G>C		Not reported	
Intron 2	c.81-14C>T		Reported (unknown)	
Intron 6	c.301+12A>C		Not reported	
Intron 6	c.302-22_302-24del		Not reported	Co-occurs with *BRCA2* pathogenic mutation (one family)
7	c.314A>G	p.(Tyr105Cys)	Reported (no)	
11	c.995G>A	p.(Arg332Gln)	Reported (unknown)	
11	c.1441C>G	p.(Leu481Val)	Not reported	Co-occurs with *BRCA1* pathogenic mutation (one family)
11	c.1971A>G	p.(=)	Reported (unknown)	
11	c.2268G>C	p.(Arg756Ser)	Reported (unknown)	
11	c.2458A>G	p.(Lys820Glu)	Reported (unknown)	
11	c.2521C>T	p.(Arg841Trp)	Reported (unknown)	
11	c.2993T>A	p.(Leu998Gln)	Not reported	
11	c.3022A>G	p.(Met1008Val)	Reported (no)	
11	c.3418A>G	p.(Ser1140Gly)	Reported (unknown)	
11	c.3739G>A	p.(Val1247Ile)	Reported (unknown)	
15	c.4535G>T	p.(Ser1512Ile)	Reported (no)	Co-occurs with *BRCA1* and *BRCA2* pathogenic mutations (two families)
Intron 15	c.4676-44A>C		Not reported	Co-occurs with *BRCA2* pathogenic mutation (one family)
16	c.4739C>A	p.(Ser1580Tyr)	Not reported	Co-occurs with *BRCA2* pathogenic mutation (one family)
16	c.4812A>G	p.(=)	Reported (unknown)	
Intron 17	c.5074+6C>G		Reported (unknown)	
18	c.5117G>C	p.(Gly1706Ala)	Reported (unknown)	
18	c.5122G>C	p.(Ala1708Pro)	Not reported	Santos et al. (7)
23	c.5411T>A	p.(Val1804Asp)	Reported (unknown)	
Intron 23	c.5468-28A>T		Not reported	
24	c.5531T>G	p.(Leu1844Arg)	Reported (yes)	
BRCA2				
2	c.2T>G	p.(Met1?)	Reported (yes)	Santos et al. (7)
3	c.125A>G	p.(Tyr42Cys)	Reported (no)	
3	c.223G>C	p.(Ala75Pro)	Reported (unknown)	
Intron 6	c.516+13C>T		Not reported	
9	c.730G>A	p.(Asp244Asn)	Reported (unknown)	
Intron 9	c.793+64dup		Not reported	
10	c.956A>C	p.(Asn319Thr)	Reported (unknown)	
10	c.1123C>T	p.(Pro375Ser)	Reported (unknown)	
10	c.1151C>T	p.(Ser384Phe)	Reported (no)	
10	c.1514T>C	p.(Ile505Thr)	Reported (no)	
10	c.1564G>C	p.(Gly522Arg)	Reported (unknown)	
10	c.1788T>C	p.(=)	Reported (unknown)	
Intron 10	c.1909+23dup		Not reported	
11	c.1938C>T	p.(=)	Reported (no)	
11	c.3226G>A	p.(Val1076Ile)	Not reported	
11	c.4068G>A	p.(=)	Reported (unknown)	
11	c.4241C>T	p.(Thr1414Met)	Reported (no)	
11	c.4258G>T	p.(Asp1420Tyr)	Reported (no)	
11	c.4321G>C	p.(Glu1441Gln)	Not reported	
11	c.4915G>A	p.(Val1639Ile)	Reported (unknown)	
11	c.4928T>C	p.(Val1643Ala)	Reported (unknown)	
11	c.5199C>T	p.(=)	Reported (no)	
11	c.5312G>A	p.(Gly1771Asp)	Reported (no)	
11	c.5645C>T	p.(Ser1882Leu)	Not reported	Co-occurs with BRCA2 pathogenic mutation (one family)

Table 2. Continued.

Exon/intron	Base change[a]	Aminoacid change	BIC[b] (clinically important[c])	Additional data
11	c.5704G>A	p.(Asp1902Asn)	Reported (no)	
11	c.5729A>T	p.(Asn1910Ile)	Not reported	
11	c.5744C>T	p.(Thr1915Met)	Not reported	Co-occurs with BRCA2 pathogenic mutation (one family)
11	c.6100C>T	p.(Arg2034Cys)	Reported (unknown)	
11	c.6210A>T	p.(Glu2070Asp)	Not reported	
14	c.7397C>T	p.(Ala2466Val)	Reported (unknown)	
14	c.7416G>A	p.(=)	Not reported	
18	c.7994A>G	p.(Asp2665Gly)	Reported (no)	
18	c.8149G>T	p.(Ala2717Ser)	Reported (no)	
19	c.8460A>C	p.(=)	Reported (unknown)	
19	c.8482A>G	p.(Ile2828Val)	Reported (unknown)	
20	c.8518A>G	p.(Ile2840Val)	Reported (unknown)	
20	c.8525G>T	p.(Arg2842Leu)	Reported (unknown)	
22	c.8850G>T	p.(Lys2950Asn)	Reported (unknown)	
22	c.8942A>G	p.(Glu2981Gly)	Not reported	
25	c.9271G>A	p.(Val3091Ile)	Reported (unknown)	
25	c.9275A>G	p.(Tyr3092Cys)	Reported (unknown)	
27	c.9720T>C	p.(=)	Reported (unknown)	
27	c.10110G>A	p.(=)	Reported (unknown)	

[a]The nomenclature of mutations is in accordance to the Human Genome Variation Society (HGVS) recommendations (version 2.121101). The DNA numbering is based on the cDNA sequences, NM_007294.3 for *BRCA1* and NM_000059.3 for *BRCA2*.
[b]BIC: Breast Cancer Information Core database (http://research.nhgri.nih.gov/bic/).
[c]Data obtained in the BIC database.

to *BRCA1* or *BRCA2* mutations in 21.4% of the 524 probands, a proportion that reaches 28.9% of the families with an *a priori* BRCAPRO mutation probability >10%. Seven additional pathogenic mutations were detected in the 526 families with BRCAPRO mutation probability <10% that were screened only for the two most frequent mutations. Thus, 119 pathogenic mutations were detected in total, of which 41.2% occurred in *BRCA1* and 58.8% in *BRCA2*. We also identified 70 different VUS, of which two (*BRCA1* c.5122G>C; *BRCA2* c.2T>G) have recently been studied in more detail (7).

Two mutations in *BRCA1* (c.2037delinsCC and c.3331_3334del) and one in *BRCA2* (c.156_157insAlu) together represent about 50% of all deleterious mutations found in Portuguese HBOC families. We have previously demonstrated that the c.156_157insAlu is a Portuguese founder mutation and that the presumed common ancestor lived about five centuries ago (1, 2). The c.2037delinsCC mutation has not been described in other populations and our preliminary haplotype study revealed that three families with the c.2037delinsCC share a common ancestor (6). The c.3331_3334del mutation has been described 43 times in the Breast Cancer Information Core (BIC) database and has been reported to occur in populations throughout the world, including Spain, Canada and Colombia (8, 9, 10). We found that families with the c.3331_3334del or the c.2037delinsCC mutations each share a common haplotype, indicating that these are founder mutations in the Portuguese population. However, the large geographical distribution of the c.3331_3334del mutation raises the question of whether it is an ancient mutation that has arisen once or whether it is a recurrent mutation that occurred independently several times, with an unrelated founder effect in Portugal and in other populations.

The identification of specific and recurrent/founder mutations in any given population allows a more efficient and cost-saving mutational screening approach. In this study, we describe a relatively straightforward and inexpensive semiquantitative multiplex PCR method that, besides allowing the simultaneous detection of the two most frequent mutations (*BRCA2* c.156_157insAlu and *BRCA1* c.3331_3334del), also avoids the preferential amplification of the normal allele (shorter PCR product). If this first-tier test is negative but the family has a mutation probability ≥10%, they should be offered additional full screening of *BRCA1* and *BRCA2* genes. In some countries, the criteria for genetic testing is based on an *a priori* >10% probability of finding a mutation based on predictive models such as BRCAPRO, BOADICEA or Manchester Score (11). We found that 19 of 112 (17.0%) pathogenic mutations found in the series of 524 fully screened probands were present in the 201 families with BRCAPRO mutation probability <10%. Of these 19 probands with deleterious mutations and a BRCAPRO mutation probability <10%, 8 (42.1%) presented one of the two most common mutations *BRCA2* c.156_157insAlu or *BRCA1* c.3331_3334del, a proportion that is similar to that found in the patients fully screened with a BRCAPRO mutation probability ≥10% (41.8%) (Fig. 2). These data indicate that it is worthwhile to test these two founder mutations in probands with clinical-criteria for genetic testing but a BRCAPRO mutation probability <10%, as demonstrated by the

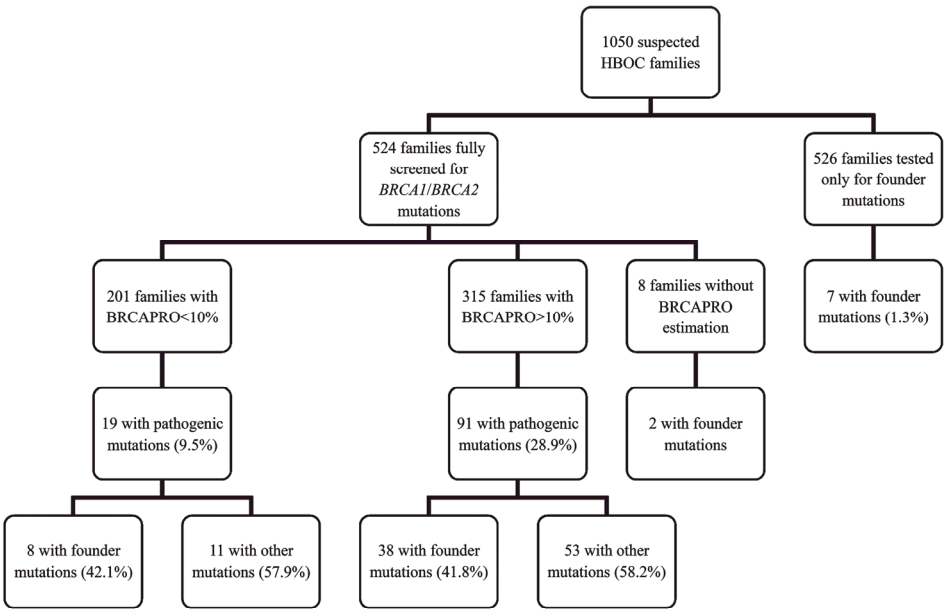

Fig. 2. Diagram showing the number of suspected HBOC families that were selected for *BRCA1* and *BRCA2* full mutation screening and for screening of the most frequent mutations in each gene. The number of positive families identified in each group is also indicated.

Table 3. Clinical-criteria for genetic testing of the most common *BRCA1/BRCA2* mutations even if *a priori* mutation probability is <10%

- Any breast cancer patient diagnosed until the age of 35 (or 40 in case of a triple negative or medullary carcinoma).
- Two first/second degree relatives with breast or ovarian cancer, at least one diagnosed before the age of 50 (or pancreatic cancer at any age).
- Three first/second degree relatives with breast, ovarian or pancreatic cancer at any age.
- Any male breast cancer case should be tested for the *BRCA2* c.156_157insAlu founder mutation.

finding of additional seven families with these common mutations in the 526 probands which were tested specifically for them. On the other hand, the likelihood of missing a deleterious mutation other than the two common founder mutations in families with BRCAPRO mutation probability <10% is about 5% (11/201). After reviewing all pedigrees with BRCAPRO mutation probabilities <10% in which deleterious mutations were found, we recommend clinical-criteria to offer genetic testing of the most common *BRCA1/BRCA2* mutations to an affected proband (Table 3).

In line with previous studies, invasive ductal carcinoma was the most common histological subtype (75%) in probands carriers of *BRCA1* and *BRCA2* germline mutations, with a relatively high frequency of medullary carcinomas in *BRCA1* carriers (19%). It is well established that a high proportion of *BRCA1*-associated breast carcinomas are estrogen receptor (ER) and progesterone receptor (PgR) negative and that tumors arising in *BRCA2* mutation carriers are more often ER and PgR positive, something that was also observed in this study. Only 5% of *BRCA1* and 10% of *BRCA2* cases were HER2 positive, which is also in accordance with literature data indicating that *BRCA1/BRCA2*-associated tumors rarely overexpress or show amplification of *HER2* (12). Despite the small number of ovarian tumors in this series, we observed that the majority of *BRCA1* ovarian cancers were invasive epithelial cancers of serous histology, which is in agreement with other reports (12). These characteristic histopathological features may be taken into consideration in cases with borderline testing criteria, both to decide testing of founder mutations or full *BRCA1/BRCA2* testing.

In conclusion, we identified a specific spectrum of germline *BRCA1/BRCA2* mutations, including three with founder effects, in Portuguese families with inherited predisposition to breast/ovarian cancer. The c.156_157insAlu *BRCA2* rearrangement and the c.3331_3334del *BRCA1* mutation are the most frequent deleterious germline mutations in Portuguese HBOC families. We therefore recommend that all suspected HBOC families from Portugal or with Portuguese ancestry living around the world, even those fulfilling moderately stringent clinical-criteria for genetic testing, should be specifically analyzed for these two mutations, and we here present a simple method for this first tier test. Screening of the entire coding regions of *BRCA1* and *BRCA2* should subsequently be offered to those families with a mutation probability ≥10% if no founder mutation is found.

Supporting Information

Additional supporting information may be found in the online version of this article at the publisher's web-site.

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Supporting Information

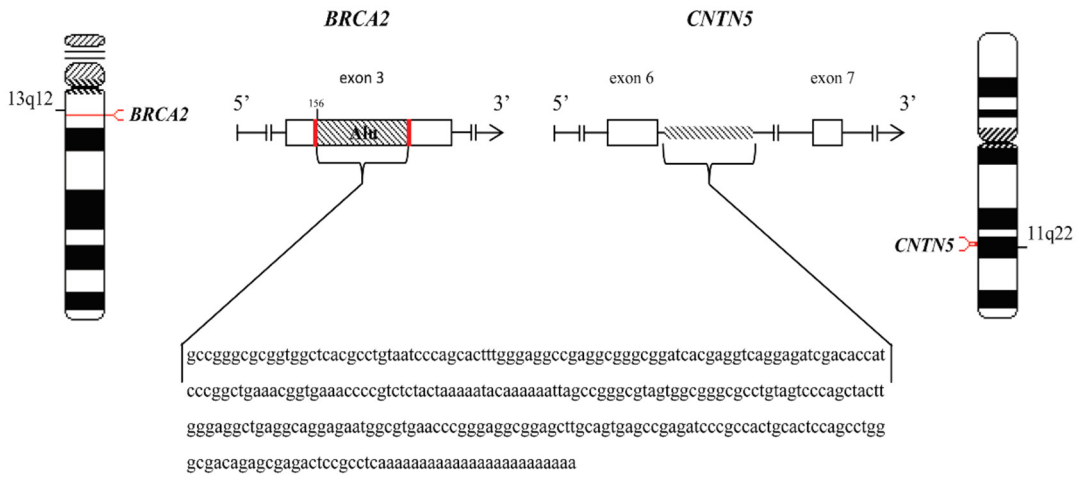

Fig. S1. Description of the AluYa5 inserted in *BRCA2* exon 3. This sequence is 100% homologous to a region in intron 6 of the *CNTN5* gene with the following nucleotide position: NC_000011.10:g.99845367_99845670; NM_014361.3:c.577+105_577+408. The Alu poly-A tail in *BRCA2* c.156_157insAlu rearrangement is predicted to be longer than the relative source element. *BRCA2* exon 3 short sequence duplication (TSD) [3] flanking the Alu element is indicated by red boxes.

Supplementary table 1 - Microsatellite marker genotypes used to reconstruct the haplotypes in probands with the c.2037delinsCC and c.3331_3334del *BRCA1* mutations.

Marker	**c.2037delinsCC[a]**							**c.3331_3334del[a]**													
	1	**2**	**3**	**4**	**5**	**6**	**7**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**
cen																						
D17S800	170	168	170	168/170	168	170	170	170	170	170	170	170	170	**170**	**170**	**170**	168/**170**	**170**	**170**/176	**170**/174	**170**/174	**170**
D17S855	141	141	**141**	**141**/149	**141**/145	**141**	**141**/147	149	149	149	149	149	149	141/**149**	**149**	147/**149**	139/**149**	145/**149**	**149**	141/**149**	139/**149**	143/**149**
D17S1323	158	158	152/**158**	152/**158**	152/**158**	152/**158**	152/**158**	152	152	152	152	152	152	**152**/160	**152**/156	**152**	**152**	**152**	**152**	**152**/158	**152**/158	148/**152**
BRCA1																						
D17S1327	160	160	**160**	128/**160**	**160**	128/**160**	128/**160**	128	128	128	128	128	128	**128**/160	**128**	**128**	**128**	**128**	**128**	**128**/160	**128**/168	**128**
D17S579	117	117	**117**	117/123	**117**	**117**	107/123	117	117	117	117	117	121	**117**/119	113/**117**	**117**/119	107/**117**	**117**	115/**117**	109/127	**117/121**	111/**117**
tel																						

[a] In the unphased haplotypes, the alleles that are consistent with the phased haplotype are indicated in bold.

Supplementary table 2 - Clinicopathological analysis of *BRCA1*/*BRCA2* breast and ovarian tumors.

Clinicopathological parameters		***BRCA1*** n (%)	***BRCA2*** n (%)	***P***
Breast tumors				
Estrogen receptor	Positive	5 (21)	41 (89)	<0.0001
	Negative	19 (79)	5 (11)	
Progesterone receptor	Positive	4 (17)	37 (80)	<0.0001
	Negative	20 (83)	9 (20)	
HER-2	Positive	1 (5)	3 (10)	NS
	Negative	19 (95)	28 (90)	
Histological type	Medullary carcinoma	5 (19)	0 (0)	0.0034
	Invasive ductal carcinoma	19 (70)	41 (78)	NS
	Invasive lobular carcinoma	2 (7)	5 (9)	NS
	Mixed ductal/lobular carcinoma	0 (0)	5 (9)	NS
	Other	1 (4)	2 (4)	NS
Ovarian tumors				
Histological type	Serous adenocarcinoma	8 (57)	1 (33)	NS
	Endometrioid adenocarcinoma	2 (14)	2 (67)	NS
	Clear cell adenocarcinoma	2 (14)	0 (0)	NS
	Other	2 (14)	0 (0)	NS

The parameters analyzed were not available for all tumors. NS, not statistically significant.

DISCUSSION

1. Contribution of the *BRCA2* c.156_157insAlu mutation to inherited predisposition to breast/ovarian cancer in families originated mostly from northern/central Portugal

The pattern of *BRCA1* and *BRCA2* mutations in 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 (Fackenthal and Olopade, 2007; Ferla et al., 2007). Identification of founder mutations makes it possible to use more specific approaches to molecular testing (Filippini et al., 2007), allowing the analysis of more patients with less stringent selection criteria in a given population. Furthermore, a frequent founder mutation allows a more accurate estimation of mutation-specific cumulative cancer incidence, facilitating also the identification of genetic and environmental risk modifiers.

A regional founder effect was described for the c.156_157insAlu *BRCA2* mutation in HBOC families mostly originated from central/southern Portugal (Machado et al., 2007). This mutation was first described by Teugels et al. (2005) in a Portuguese breast cancer patient living in Belgium (Teugels et al., 2005).

We estimated the c.156_157insAlu mutation frequency in breast/ovarian cancer families originated mostly from northern/central Portugal and identified eight probands with the c.156_157insAlu mutation in our initial series of 78 patients in whom no deleterious *BRCA1*/*BRCA2* mutation had been found, corresponding to a frequency of about 8% in families suspected to have hereditary predisposition to breast/ovarian cancer (Paper I). This finding, together with the 22 pathogenic mutations previously reported in that series (Peixoto et al., 2006), raised the deleterious mutation detection rate to 30% (30/100) of all cases studied (Paper I). When we enlarged our series to 524 Portuguese breast/ovarian cancer families, inherited cancer predisposition could be linked to *BRCA1* or *BRCA2* mutations in 21.4% of the fully screened probands, a proportion that reached 28.9% of the families with an *a priori* BRCAPRO mutation probability >10% (paper V). This mutation rate is slightly above what has been reported in the majority of populations using similar criteria for referral to genetic

counseling and testing (Shih et al., 2002; Diez et al., 2003; Perkowska et al., 2003; Claes et al., 2004), something that might reflect the fact that we are now able to detect a mutation that escaped conventional mutation screening techniques and that it represents 57.1% of all *BRCA2* mutations and 33.6% of all *BRCA1*/*BRCA2* mutations (Paper V). It also indicates that other *BRCA1*/*BRCA2* mutations undetectable by current-day screening methodologies may remain to be uncovered in high-risk breast/ovarian cancer families.

2. Ancestral origin and population spread of the c.156_157insAlu *BRCA2* mutation

Although all reported c.156_157insAlu *BRCA2* mutations have so far been identified in Portuguese HBOC families, and since this mutation is not detectable using the common screening methodologies, one could not exclude that it was present in other populations until it was 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 5,294 suspected HBOC families with no known deleterious *BRCA1*/*BRCA2* mutations from several countries mainly from Europe, but also from Asia and North and South America (Paper III). We only 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, the 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 finding of a common ancestry for the Portuguese HBOC families presenting the R71G *BRCA1* founder mutation of Galician origin (Santos et al., 2009).

Machado et al. (2007) claimed that the c.156_157insAlu *BRCA2* mutation had its origin 2,400–2,600 years ago in what is now central/southern Portugal (Machado et al., 2007). 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 estimated by Machado et al. (Machado et al., 2007). 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. For instance, the mutation age estimate for the *BRCA1* mutation c.5266dupC originally described as a founder mutation in the Ashkenazi Jewish population, but that is also present in several European countries, revealed that it arose about 1800 years ago in either Scandinavia or what is now northern Russia. Subsequently this mutation spread to various populations during the following centuries, including the Ashkenazi Jewish population approximately 400–500 years ago (Hamel et al., 2011). In order to get a more accurate mutation age estimate of the c.156_157insAlu *BRCA2* rearrangement, we performed an extensive haplotype analysis in 11 informative families and assuming a generation time of 25 years, we estimated the age of the c.156_157insAlu *BRCA2* mutation to be 558 ± 215 years (Paper III). Our estimate is consistent with the widespread distribution of the mutation in Portugal (Machado et al., 2007) (Papers I, III, and V), 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). On the other hand, although the mutation was initially reported in Portugal and in a few families with Portuguese ancestry living in Belgium, France or the USA, we cannot 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. One such example is the subsequent identification of this mutation in three unrelated breast cancer patients originated from Brazilian families, all of them with family names of Portuguese origin and with a haplotype compatible with that found in the Portuguese *BRCA2* HBOC families carrying the c.156_157insAlu mutation (Moreira et al., 2012).

Since the c.156_157insAlu *BRCA2* mutation is not readily detectable by regular or next-generation mutation screening (Teugels et al., 2005; De Brakeleer et al., 2013) and there are millions of Portuguese emigrants and individuals with Portuguese ancestry with a worldwide distribution (Observatório da Emigração, 2014), it is very important to call attention to the necessity of specifically testing for this mutation in such cases. This has recently been well illustrated by Benson

et al. (2014), who reported that approximately 8% of the Rhode Island population in the USA claim Portuguese ancestry and who recommended that any breast cancer patient with Portuguese ancestry having a family history suggestive of HBOC should have *BRCA2* c.156_157insAlu mutation testing and that those patients who were tested years ago by sequencing should return for updated testing (Benson et al., 2014).

3. Characterization of the pathological and clinical significance of the c.156_157insAlu *BRCA2* mutation

It is important to determine whether mutations are disease causing or merely represent rare variants associated with no or little cancer risk that will generate significant problems in risk evaluation, counseling, and adoption of prophylactic measures. Some criteria are generally accepted to ascertain the pathogenicity of *BRCA1*/*BRCA2* variants, namely, family-based segregation and cancer history; coexistence of variants with pathogenic mutations in the same gene (assuming that *BRCA1* biallelic deleterious mutations are embryonically lethal and that those of *BRCA2* are either lethal or produce a Fanconi anemia phenotype); the frequency of the variants in unaffected controls; alterations located in highly conserved functional domains and variants that affect mRNA processing (Chenevix-Trench et al., 2006; Farrugia et al., 2008).

The c.156_157insAlu mutation consists in an Alu element insertion of the AluYa5 subtype in the *BRCA2* exon 3 (Teugels et al., 2005) that is 100% homologous to a region in intron 6 of the *CNTN5* gene (Paper V). Teugels et al. (2005) demonstrated that this mutation originates an in-frame deletion of exon 3 from the *BRCA2* mRNA encoding a transcriptional activation domain (Teugels et al., 2005). On the other hand, Díez et al. (2007) issued a word of caution when interpreting as pathogenic alterations causing *BRCA2* exon 3 skipping, as exon 3 splicing can also be detected in normal tissues (Teugels et al., 2005; Diez et al., 2007). In order to better characterize the pathological and clinical significance of this mutation, we have looked for this mutation in disease-free controls, analyzed in more detail the mRNA in carriers and noncarriers, performed cosegregation of the mutation with disease and evaluated the cumulative incidence of breast cancer in c.156_157insAlu *BRCA2* mutation carriers. Four separate findings corroborate the conclusion that the c.156_157insAlu *BRCA2*

mutation is indeed deleterious. First, *BRCA2* full length transcript is derived only from the wild type allele in patients carrying the c.156_157insAlu *BRCA2* mutation, whereas the *BRCA2*-Δex3 transcript was transcribed mostly from the mutated allele. We evaluated the relative contribution of each allele to normal and *BRCA2*-Δex3 transcripts in patients and controls, the latter showing both alleles giving rise to a small proportion of the *BRCA2*-Δex3 transcript (Paper I and II). We also demonstrated by quantitative real-time polymerase chain reaction (PCR) that carriers present significantly more *BRCA2*-Δex3 transcripts and much less full length transcripts than controls (Paper III). Second, if the abnormal transcript with the in-frame deletion of exon 3 is fully functional, as it was suggested by Díez et al. (2007), then this variant would have to be considered a polymorphism given its high frequency (8%) in families suspected to have predisposition to HBOC (Diez et al., 2007). However, we did not find the c.156_157insAlu in 262 chromosomes from healthy blood donors, allowing us to dismiss the hypothesis that it is a neutral polymorphism (Paper I). Third, we observed co-segregation of this alteration and disease (Paper I). Fourth, 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 or elsewhere (Ford et al., 1998), further strengthening its role as the major contributor to hereditary predisposition to breast cancer in Portugal (Paper III). Although the function, if any, of the *BRCA2* exon 3 skipping seen in controls is unknown, the observed breast cancer risk in c.156_157insAlu carriers would not be expected if this transcript was fully functional.

4. Molecular characterization of large genomic rearrangements detected in Portuguese HBOC families

According to Sluiter and van Rensburg (2011), 81 and 17 different LGRs have been characterized for the *BRCA1* and *BRCA2* genes, respectively (Sluiter and van Rensburg, 2011). The high content of Alu elements in the intronic sequences of both *BRCA1* and *BRCA2* genes contributes to the occurrence of rearrangements in these genes, not only because Alu sequences are known to mediate the occurrence of genetic recombination events as a direct result of their abundance and sequence homology, but also due to the occurrence of insertion

mutations as a consequence of Alu retrotransposition events (Smith et al., 1996; Welcsh and King, 2001). The detection of LGRs requires specific techniques, like multiplex ligation probe amplification (MLPA) or quantitative PCR, however they only give information about the exonic region involved in the rearrangement and not on the exact genomic breakpoints (van den Ouweland et al., 2009). To determine whether a LGR is actually novel and to gain insight about the mutational mechanism responsible for its occurrence, molecular characterization with breakpoint identification is mandatory. We characterized two deletions involving exons 1 to 7 (c.-232-?_441+?del) and exons 11 to 15 (c.671-?_4675+?del) of the *BRCA1* gene, both detected by MLPA (Paper IV). The first rearrangement harbored a deletion comprising exon 7 of the *BRCA1* gene to exon 11 of the *NBR1* gene (c.441+1724_o*NBR1*:c.1073+480del). Two highly homologous Alu elements were found in the genomic sequences flanking the deletion breakpoint, namely, an AluSc in *BRCA1* intron 7 and an AluSg in *NBR1* intron 11. The alignment of these intronic sequences suggested that the most likely mechanism for the occurrence of this rearrangement was nonallelic HR between these Alu repeats, substantiated by the presence of a 20-bp overlapping sequence at the breakpoint junction.

The second rearrangement fully characterized at the nucleotide level was the *BRCA1* mutation c.671-319_4677-578delinsAlu. The 3' breakpoint occurred in an AluY element and the 5' breakpoint was 120 bp downstream from a second Alu element (AluJb) in the *BRCA1* gene. In addition, an ~250-bp Alu fragment was inserted at the breakpoints of the deleted region. This Alu fragment presents a high homology to the intron 15 breakpoint region of the *BRCA1* gene and is an Alu element belonging to the AluY family. Alu mediated unequal HR is the most common mechanism causing *BRCA1* LGRs. The deletion/insertion event here described could be due to HR with exchange of genetic material (Slebos et al., 1998). However, another possible explanation for the observed rearrangement could involve an insertion-mediated deletion mechanism caused by the retrotransposition of Alu elements at this genomic site leading to the deletion of adjacent genomic regions (Slebos et al., 1998; Gilbert et al., 2002; Chen et al., 2005; Okubo et al., 2007). This hypothesis is corroborated by the fact that the inserted Alu element belongs to a still active AluY family. Additionally, the sequence flanking the c.671-319_4677-578delinsAlu rearrangement breakpoint

was analyzed by Hancks and Kazazian (2016), and included in a recent review of human retrotransposition insertions in human diseases (Hancks and Kazazian, 2016). The deletion of exons 11–15 of the *BRCA1* gene was also described by de la Hoya et al. (2006), but the mutational mechanism and the genomic breakpoint (L78833:g.33450_56562del) are different from those we here report, emphasizing the importance of breakpoint characterization to determine whether rearrangements involving the same exons are related or have occurred independently (de la Hoya et al., 2006).

5. Mutational spectrum characterization and evaluation of the impact of founder mutations in the genetic testing criteria and strategy for molecular testing of HBOC families of Portuguese ancestry

The first reports on the prevalence of *BRCA1* and *BRCA2* germline mutations in Portugal were based on the study of relatively small series of families suspected of inherited predisposition to breast/ovarian cancer. Duarte *et al* (2002) analyzed 76 families from northern Portugal and Galicia with family history of breast and/or ovarian cancer and reported that 6.5% presented pathogenic mutations, whereas Machado *et al.* (2007) detected 19 (36%) deleterious mutations in the 53 probands fully screened for *BRCA1* and *BRCA2* mutations (using selection criteria of BRCAPRO mutation probability >25%) (Duarte et al., 2002; Machado et al., 2007).

We characterized the mutational spectrum of germline *BRCA1*/*BRCA2* mutations in 1050 Portuguese breast/ovarian cancer families, of which 524 were fully screened (Paper V). Inherited cancer predisposition could be linked to *BRCA1* or *BRCA2* mutations in 21.4% of the 524 fully screened probands, a proportion that reaches 28.9% of the families with an *a priori* BRCAPRO mutation probability >10%. Seven additional pathogenic mutations were detected in the 526 families with BRCAPRO mutation probability <10% that were screened only for the two most frequent mutations. Thus, 119 pathogenic mutations were detected in total, of which 41.2% occurred in *BRCA1* and 58.8% in *BRCA2*. We also identified 70 different VUS, of which two (*BRCA1* c.5122G>C; *BRCA2* c.2T>G) have been studied in more detail (Santos et al., 2014).

Two mutations in *BRCA1* (c.2037delinsCC and c.3331_3334del) and one in *BRCA2* (c.156_157insAlu) together represent about 50% of all deleterious

mutations found in Portuguese HBOC families (Paper V). The *BRCA2* rearrangement is responsible for more than half of all deleterious *BRCA2* mutations and about one-third of all deleterious mutations in HBOC families. The c.2037delinsCC mutation has not been described in other populations and our preliminary haplotype study revealed that three families with the c.2037delinsCC share a common ancestor (Peixoto et al., 2006). The c.3331_3334del mutation has been described 43 times in the Breast Cancer Information Core (BIC) database and has been reported to occur in populations throughout the world, including Spain, Canada and Colombia (Durocher et al., 1996; Blesa et al., 2000) (Torres et al., 2007). We found that families with the c.3331_3334del or the c.2037delinsCC mutations each share a common haplotype, indicating that these are founder mutations in the Portuguese population. However, the large geographical distribution of the c.3331_3334del mutation raises the question of whether it is an ancient mutation that has arisen once or whether it is a recurrent mutation that occurred independently several times, with an unrelated founder effect in Portugal and in other populations.

The identification of specific and recurrent/founder mutations in any given population allows a more efficient and cost-saving mutational screening approach. We developed a relatively straightforward and inexpensive semi-quantitative multiplex PCR method that, besides allowing the simultaneous detection of the two most frequent mutations (*BRCA2* c.156_157insAlu and *BRCA1* c.3331_3334del), also avoids the preferential amplification of the normal allele (shorter PCR product) in c.156_157insAlu carriers (Paper V). If this first-tier test is negative but the family has a mutation probability ≥10%, they should be offered additional full screening of *BRCA1* and *BRCA2* genes. In some countries, the criteria for genetic testing is based on an ***a priori*** >10% probability of finding a mutation based on predictive models such as BRCAPRO, BOADICEA or Manchester Score (Balmana et al., 2011). We found that 19 of the 112 (17.0%) pathogenic mutations found in the series of 524 fully screened probands were present in the 201 families with BRCAPRO mutation probability <10%. Of these 19 probands with deleterious mutations and a BRCAPRO mutation probability <10%, eight (42.1%) presented one of the two most common mutations *BRCA2* c.156_157insAlu or *BRCA1* c.3331_3334del, a proportion that is similar to that found in the patients fully screened with a BRCAPRO mutation probability ≥10%

(41.8%). These data indicate that it is worthwhile to test these two founder mutations in probands with clinical criteria for genetic testing but a BRCAPRO mutation probability <10%, as demonstrated by the finding of additional seven families with these common mutations in the 526 probands which were only tested specifically for them. On the other hand, the likelihood of missing a deleterious mutation other than the two common founder mutations in families with BRCAPRO mutation probability <10% is about 5% (11/201). After reviewing all pedigrees with BRCAPRO mutation probabilities <10% in which deleterious mutations were found, we recommend offering genetic testing of the most common *BRCA1*/*BRCA2* mutations to an affected proband according to the following clinical criteria (Paper V):

- Any breast cancer patient diagnosed until the age of 35 (or 40 in case of a triple negative or medullary carcinoma);
- Two first/second degree relatives with breast or ovarian cancer, at least one diagnosed before the age of 50 (or pancreatic cancer at any age);
- Three first/second degree relatives with breast, ovarian or pancreatic cancer at any age;
- Any male breast cancer case should be tested for the *BRCA2* c.156_157insAlu founder mutation.

Current guidelines for *BRCA1*/2 genetic testing require that a significant personal or family history of cancer be present to be eligible for testing. However, a proportion of women with mutations are missed using the current guidelines (Hartge et al., 1999; Metcalfe et al., 2010; Manchanda et al., 2015b). Population-based genetic testing for *BRCA1*/2 founder mutations have been conducted in defined population subgroups, like Ashkenazi Jewish populations, independently of personal or family history of cancer. This strategy proved to be cost-effective in the Ashkenazi Jewish populations due to the mutational pattern (three mutations comprise the majority of deleterious mutations in the Jewish population) and the high frequency of these mutations in this population (Manchanda et al., 2015a; Foulkes et al., 2016). Extending population-based genetic testing to other populations has, however, many obstacles that are currently in debate. Our strategy to offer genetic testing of the Portuguese *BRCA1*/2 founder mutations to families with certain clinical characteristics but

without criteria for full analysis, although not being population-based genetic testing, also aims for a more-comprehensive and cost-effective detection of mutation carriers.

We therefore recommend that all suspected HBOC families from Portugal or with Portuguese ancestry living around the world, even those fulfilling moderately stringent clinical criteria for genetic testing, should be specifically analyzed for *BRCA2* c.156_157insAlu and *BRCA1* c.3331_3334del mutations. Screening of the entire coding regions of *BRCA1* and *BRCA2* should subsequently be offered to those families with a mutation probability ≥10% if no founder mutation is identified (Paper V).

CONCLUSIONS

The main conclusions of this thesis are:

1. Inherited cancer predisposition could be linked to *BRCA1* or *BRCA2* mutations in about 30% of the Portuguese breast/ovarian cancer families.
2. Despite the heterogeneous mutational spectrum of germline *BRCA1*/*BRCA2* mutations in Portuguese breast/ovarian cancer families, three variants only (c.156_157insAlu in *BRCA2* and c.3331_3334del and c.2037delinsCC in *BRCA1*) account for about 50% of all pathogenic mutations.
3. The *BRCA2* c.156_157insAlu rearrangement is a Portuguese founder mutation originated about 558 ± 215 years ago and has so far only been reported in HBOC families from Portugal or of Portuguese ancestry.
4. The c.156_157insAlu mutation originates *BRCA2* exon 3 skipping and the *BRCA2* full length transcript is derived only from the wild type allele in carriers, with several lines of evidence showing that the c.156_157insAlu *BRCA2* mutation is deleterious.
5. Families with the *BRCA1* c.3331_3334del or c.2037delinsCC mutations each share a common haplotype, indicating that these are also founder mutations in the Portuguese population.
6. The breakpoints of two novel large deletions involving the *BRCA1* gene were identified, with both rearrangements most likely being mediated by Alu elements, either by HR between Alu repeats or by the retrotransposition of an Alu element.
7. As the two most common mutations c.156_157insAlu in *BRCA2* and c.3331_3334del in *BRCA1* were found in a significant proportion of the HBOC families with an *a priori* BRCAPRO mutation probability <10%, we recommend offering genetic testing of these mutations to any proband with well-defined clinical criteria, which should be followed by screening of the entire coding regions of *BRCA1* and *BRCA2* in those families with a mutation probability ≥10%.

FUTURE PERSPECTIVES

The work of this thesis has raised several new questions that will be addressed in the near future, namely:

1. We observed that families with the *BRCA1* c.3331_3334del mutation share a common haplotype, indicating that this is a founder mutation in the Portuguese population. However, the large geographical distribution of the c.3331_3334del mutation raises the question of whether it is an ancient mutation that has arisen once or whether it is a recurrent mutation that occurred independently several times, with an unrelated founder effect in Portugal and in other populations. To address this question, haplotype analysis will be performed in carriers of this mutation from Portugal and from different populations where this mutation has been described recurrently.
2. We aim to optimize the semi-quantitative multiplex assay that detects the *BRCA2* c.156_157insAlu and *BRCA1* c.3331_3334del mutations to include the third most common mutation (*BRCA1* c.2037delinsCC) in order to identify 50% of all mutations found in Portuguese HBOC families with a simple and cheap assay.
3. Since deleterious germline mutations are found in less than 30% of the breast/ovarian cancer families with criteria for genetic testing, we will perform panel gene testing with next-generation sequencing technology in the *BRCA1*/*BRCA2* negative cases in order to investigate the contribution of other genes for inherited predisposition.

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