EPIDEMIOLOGY

BRCA1/BRCA2 mutation status and clinical-pathologic features of 108 male breast cancer cases from Tuscany: a population-based study in central Italy

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Abstract *Background* Male breast cancer (MBC) is a rare and scarcely investigated disease. The strongest genetic risk factor for MBC is represented by inherited *BRCA2* mutations, whereas the association between MBC and *BRCA1* mutations is less clear. MBC appears to be biologically similar to breast cancer in females, however the phenotypic characteristics of *BRCA1/2*-related MBCs are not yet well elucidated. *Objective* To investigate the genetic and phenotypic characteristics of MBC in a large and well-characterized population-based series of 108 MBCs from Tuscany (Central Italy) and to evaluate associations between *BRCA1/BRCA2* mutation status and clinical-pathological features including breast/ovarian cancer first-degree family history, tumor histology and grade, proliferative activity, estrogen/progesterone receptors (ER/PR) and epidermal growth factor receptor 2 (HER2) expression. *Results* *BRCA1/BRCA2* mutations were identified in ten MBCs, in particular, two cases (1.9%) carried *BRCA1* and eight cases (7.4%) carried *BRCA2* mutations. The same *BRCA1* mutation (3347delAG) was detected in two unrelated MBC cases. Three novel *BRCA2* pathogenic mutations were found. Statistically significant associations emerged between *BRCA2*-related tumors and absence of PR expression ($P = 0.008$), HER2 over-expression ($P = 0.002$) and high tumor grade ($P = 0.005$). *Conclusions* Here, we (i) reported that in our population about 9% of MBC cases are accounted for by *BRCA1/BRCA2* mutations; (ii) enlarged the *BRCA2* mutational spectrum and (iii) characterized a specific phenotype associated with *BRCA2*-related MBCs suggestive of aggressive behavior. Overall, our results may have important implications on clinical management for this rare disease.

Introduction

Male breast cancer (MBC) is a rare disease, accounting for <1% of all cancers in men. However, recent epidemiological studies indicate that its incidence is rising 1.1% annually [1, 2]. In Europe, the incidence of MBC is 1 in 100,000 men per year, and <1% of all breast cancer (BC) patients are male [3]. In Italy, MBC accounts for 0.2% of all cancers in males and incidence rates, standardized on the European population, are about 1 new case $\times$ 100,000 men per year [4].

At the epidemiological level, male and female BC share many similarities with the main differences represented by a lower incidence and older age of occurrence of MBC [5]. Hormonal, environmental and genetic factors are involved in the pathogenesis of BC in women as well as in men. Major risk factors related to MBC predisposition include pathological conditions associated with primary and secondary hyper-estrogenism, radiation exposure, and, particularly, a family history (FH) positive for breast and/or ovarian cancer. About 20% of MBC patients have a first-degree relative

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affected by BC [3], which points to a relevant genetic component in MBC predisposition. Germ-line mutations of *BRCA2* (OMIM # 600185) and, with lower frequency, of *BRCA1* (OMIM # 113705) are implicated in MBC predisposition. However, the frequency of *BRCA1* and *BRCA2* mutations are quite different in ethnically diverse population- and clinic-based MBC series, ranging between 4% and 40% for *BRCA2* and up to 4% for *BRCA1,* being higher in the presence of founder effects [6]. *BRCA1* and *BRCA2* founder mutations have been identified in specific countries or ethnic groups, particularly in genetically isolated populations such as the Icelanders and Ashkenazi Jews. In Italy, there is evidence of founder *BRCA1* and *BRCA2* mutations in regions that show a micro-homogeneity [7–11]. As for the association between MBC and *BRCA2* germ-line mutations, the presence of a male affected by BC seems to be the strongest predictor for the occurrence of *BRCA2* genomic rearrangements in high-risk families. Indeed, *BRCA2* rearrangements are observed in about 10% of MBC families negative for *BRCA1/2* point mutations, whereas *BRCA1* rearrangements are rarely found [12–16]. On the other hand, we recently reported that both *BRCA2* and *BRCA1* rearrangements are infrequent in MBC cases, negative for *BRCA1/2* mutations, unselected by FH [17].

Male breast cancer and female breast cancer (FBC) appear to be biologically similar but they differ mainly in the frequency of histological types and in the expression of hormone receptors (estrogen/progesterone, ER/PR) and of epidermal growth factor receptor 2 (HER2). In MBC, the predominant histological type is invasive ductal carcinoma, which characterizes more than 90% of all MBC cases [18]. MBC shows higher rates of ER/PR positivity (ER/PR+) and lesser rate of HER2 over-expression (HER2+), compared with FBC [3, 5, 6]. Accumulating evidence supports that *BRCA1* and *BRCA2*-related FBC tend to manifest specific phenotypic profiles [19]. In particular, *BRCA1*-tumors tend to be ER-, PR- and HER2-negative (triple negative) and *BRCA2*-tumors tend to be more often ER/PR+ and HER2− [20]. At present, very few data are available for *BRCA1/2*-related MBC [21]. Indeed, due to its rarity, MBC is less investigated compared to FBC, but it is now receiving more attention, probably because of its increasing incidence and of the better knowledge on the role of *BRCA* genes mutations in MBC predisposition. Retrospective studies on large series of MBC have been recently performed to investigate the major clinical-pathologic characteristics of BC in men [18, 22]; however, these studies did not report data on *BRCA1/2* mutation status. On the other hand, studies concerning *BRCA1/2* mutation screening are often limited in the number of cases analyzed. To our knowledge, very few studies have been performed in MBC series of more than 100 cases and the screening was limited to founder mutations [23, 24].

The present study was carried out with the aim to characterize the genetic and phenotypic features of MBC from a large and epidemiologically well-characterized population-based series from Tuscany (Central Italy). In particular, we wished (i) to assess the prevalence and spectrum of *BRCA1* and *BRCA2* mutations and (ii) to investigate the phenotypic features of tumors in order to further characterize *BRCA1/2*-related MBCs.

Patients and methods

MBC patients

In the present study, we expanded the original population-based series of 25 MBC cases diagnosed in the area of Florence (Tuscany, Central Italy) in the period 1990–1998, that we have previously described [25], based on all currently available local sources (including Pathology Departments and the Hospital Discharge database). We enrolled 83 additional MBC cases, diagnosed in the period 1991–2007, for a total of 108 MBCs residing in Eastern Tuscany. The recruitment of the new MBC cases was carried out according to the same protocol as used for the previous MBC series. Overall, after exclusion of deceased and migrated patients, 94 additional unrelated MBC were traced and invited to participate into the study. Eleven cases refused to participate, mostly because of advanced age or severe illness, thus confirming our previous high response rate (83/94, 88.3%). For each study participant we obtained: (i) a signed informed consent form with a detailed description of the study protocol, including the information about the mutational analysis of the *BRCA1/BRCA2* genes; (ii) detailed information on his FH for cancer at any sites, including all first- and second-degree relatives of both genders (all of this information was validated by available sources, mainly local Cancer and Mortality Registries); (iii) detailed information on his personal history of cancer at any site; (iv) information on life style habits and a detailed occupational history up to the date of BC diagnosis; (v) one or more blood sample(s). All information were collected by a genetician in the frame of the High-Risk Cancer Family Project. The a priori probability of carrying a mutation in the *BRCA1/2* genes was estimated by using the BRCAPRO software version 4.2 [26]. Pathological reports and histological slides for all of the MBC cases were identified and retrieved from the archival files of the Pathology Departments of the area for diagnostic confirmation. Procedures to maintain confidentiality for all the information collected were developed and strictly applied. The study was approved by the Florence Ethical Research Committee.

Mutational analysis

The entire *BRCA1* and *BRCA2* coding sequences were screened for germ-line mutations. After isolation of genomic DNA from buffy coats, PCR-amplified *BRCA1* exons 2–10 and 12–24 and *BRCA2* exons 2–9 and 12–27 were analyzed by combining single strand conformational polymorphism (SSCP) and automatic sequencing with the ABI Prism-310 Genetic Analyzer (Applied Biosystems, Warrington, UK). The larger exons (*BRCA1* exon 11, *BRCA2* exons 10 and 11) were rapidly screened for truncating mutations by the protein truncation test (PTT) or by automatic sequencing analysis in cases negative for truncating mutations within these exons. Mutations were verified on two independent blood samples. The DNA mutation nomenclature is as used in the Breast Information Core (BIC) database (http://research.nghgri.nih.gov/bic) according to GenBank, U14680.1 and U43746 for *BRCA1* and *BRCA2*, respectively. Novel *BRCA1/2* variant sequences were analysed in silico for predicted splicing defects by using SpliceSiteFinder (http://www.genet.sickkids.on.ca/~ali/spilcesitefinder.html) and ESEfinder (http://rulai.cshl.edu/tools/ESE3/). Intronic variants predicted to alter RNA splicing were further analysed by RT-PCR, carried out using Superscript II (Invitrogen, Carlsbad, CA, USA) reverse transcriptase with gene-specific primers for the cDNA. Total RNA was extracted from peripheral blood leukocytes by using Trizol Reagent and following manufacturer instructions (Invitrogen, Carlsbad, CA, USA).

Haplotype analysis

A total of 10 individuals belonging to the two families segregating the recurrent *BRCA1* 3347delAG mutation and 14 population controls were genotyped at six microsatellite markers, including three intragenic loci (D17S1323, D17S1322 and D17S855, located in *BRCA1* intron 12, 19 and 20, respectively), one centromeric (D17S250) and two telomeric (D17S858 and D17S1183) markers. The haplotype analysis encompassed a distance of about 1,332 Kb between the D17S855 and D17S1183 markers. Alleles were numbered according to the size of each microsatellite repeat marker. The disease-associated haplotypes were deduced by inspection of segregating genotypes in the analyzed individuals.

Immunohistochemical analysis

Step sections from formalin-fixed, paraffin-embedded primary mammary tumor blocks were obtained for the majority of the cases in order to perform ad hoc immunohistochemical analyses. Overall, out of 110 tumors in 108 patients, we obtained tissue blocks for 96 tumors in 94 patients (including two blocks each for two bilateral MBCs and only one for the other bilateral case). Immunohistochemical studies were performed on 96 available formalin-fixed paraffin-embedded tissue blocks of 94 patients (out of the original 108 cases; including 5 blocks of 3 patients with bilateral BC). Immuno-histochemical data could not be obtained for some of these MBCs because available tissue specimens were not sufficient for the whole study protocol. Markers were thus available for a variable number of tumors (range 90–96). The following primary antibodies were applied: anti-HER2, clone TAB 250 (Zymed, S. Francisco, CA), diluted 1:20; anti-ER, clone 6F11 (Ventana Medical Systems, Tucson, AZ) prediluted; anti-PR, clone 1A6 (Ventana Medical Systems, Tucson, AZ) prediluted and anti-Ki-67, clone MIB1 (Dako Cytomation, Glostrup, DK) diluted 1:60. Immunoistochemical protocols and scoring procedures have been described in detail elsewhere [25, 27].

Statistical analysis

The association between mutation carrier status and first-degree FH of breast/ovarian cancer was measured by calculating the prevalence rate ratio and its 95% CI. The association between mutation carrier status and PR, ER, HER2 or MIB1 immuno-histochemical expression was tested by using the Fisher exact test. The association between mutation carrier status and histopathological grade was tested using the Wald test of the coefficient associated with histopathological grade and considered as continuous variables in a logistic regression analysis with mutation carrier status as outcome. A P value <0.05 was considered statistically significant. All the analyses were carried out by using the SAS statistical software (SAS/STAT version 9.1).

Results

Prevalence and spectrum of *BRCA1* and *BRCA2* mutation in a population-based series of Italian MBC cases

To evaluate the contribution of *BRCA1* and *BRCA2* mutations in MBC we enrolled a large and well-characterized population-based series of 108 MBC cases, diagnosed in the period 1991–2007 in the area of Florence (Tuscany, Central Italy), thus expanding our previous study based on 25 MBCs [25]. In the present population-based series, age at first BC diagnosis ranged between 24 and 90 years (mean: 63.2 years; median: 65.0 years). Overall, 25.9% of the patients reported a first-degree FH of breast/ovarian cancer (Table 1), including one patient with a first-degree FH of MBC. Furthermore, 37 patients (34.3%) had

a first-degree FH of cancer at sites other than breast/ovary, including nine patients (8.3%) that reported both a first-degree FH of breast/ovarian cancer and of cancer at other sites. A personal history of cancer(s) at sites other than breast was reported in 14.8% of MBC cases (Table 1). In these patients prostate and bladder carcinomas were the most frequently observed metachronous cancers. Notably, three out of 108 MBC patients had been diagnosed with bilateral BC. With regard to the pathological characteristics of the MBC cases (Table 1), the majority of the tumors was invasive ductal carcinomas (78.2%), expressed ER (ER+, 87.5%) and PR (PR+, 79.2%), showed low proliferative activity (MIB1−, 65.6%) and did not over-express HER2 (HER2−, 81.3%).

Overall, deleterious BRCA1/2 germ-line mutations were detected in 10 of the 108 MBC cases analyzed, with a mutation prevalence of 9.3% (95% CI, 4–15%). In particular, two cases (1.9%, 95% CI, 0–4%) carried BRCA1 and eight cases (7.4%, 95% CI, 2–12%) carried BRCA2 mutations (Table 2). The same BRCA1 mutation (3347delAG) was identified in two unrelated MBC cases (Fig. 1). Haplotype analysis allowed the identification of a common haplotype shared by all mutation carriers (seven individuals) but not by non carrier relatives (three individuals). The same analysis conducted in 14 healthy controls revealed strong allele variability and different allele pattern compared to the carriers belonging to the BRCA1 3347delAG families (data not shown). As shown in Table 2, of the eight BRCA2 mutations here identified three are novel mutations not reported in the BIC database (http://research.nghgri.nih.gov/bic). Two of them, the BRCA2 802insA and the BRCA2 7935delT, are frameshift mutations that create a stop at codons 205 and 2647, respectively (Table 2, Fig. 2). The third novel mutation, the BRCA2 IVS7-2 A>G, is an intronic variant. By in silico analysis, the BRCA2 IVS7-2 A>G is predicted to affect the splicing of exon 8 by disrupting the intron 7 acceptor site (Splice-SiteFinder wild type efficiency score = 93.4) and the exonic splice enhancer SRp40 motif (ESEfinder wild type efficiency score = 3.63). We evaluated the consequence of the BRCA2 IVS7-2 A>G variant on splicing in vivo by examining RNA isolated from peripheral lymphocytes of the MBC patient (MB 115) carrying the variant. RT-PCR, performed to amplify a region encompassing BRCA2 exons 6–9 produced two cDNA fragments: one of the predicted size (314 bp) for the wild type product and the other approximately 50 bp smaller than the wild type cDNA product (Fig. 3). Direct sequence analysis of the abnormal PCR product showed a transcript lacking exon 8 (BRCA2 860–909del) and containing a stop signal at codon 236.

By using the BRCAPRO software version 4.2 [26], the a priori probability of carrying a mutation in BRCA1/2 was estimated for all cases (mean value: 23.8%; range: 0–100%).

Table 1 Clinico-pathological characteristics of 110 tumors from the population-based series of 108 Italian MBC cases (Florence 1990–2007)

Clinico-pathological characteristics	N (%)[a]
Family history of breast/ovarian cancer	
Negative	80 (74.1)
Positive	28 (25.9)
Total	108
Personal history of cancer	
Negative	92 (85.2)
Positive	16 (14.8)
Total	108
Histology	
Invasive ductal carcinoma	86 (78.2)
In situ ductal carcinoma	8 (7.3)
Papillary carcinoma	6 (5.5)
Mucinous (colloid)	1 (0.9)
Lobular carcinoma	3 (2.7)
Paget's	2 (1.8)
Adenoid cystic carcinomas	2 (1.8)
Cribriform carcinoma	2 (1.8)
Total	110
Grade	
G1	18 (19.0)
G2	50 (52.6)
G3	27 (28.4)
Total	95
Stage	
0	10 (12.0)
I	31 (37.4)
II	30 (36.1)
III–IV	12 (14.5)
Total	83
ER	
Negative	12 (12.5)
Positive	84 (87.5)
Total	96
PR	
Negative	20 (20.8)
Positive	76 (79.2)
Total	96
MIB1	
Negative	59 (65.6)
Positive	31 (34.4)
Total	90
HER2	
Negative	74 (81.3)
Positive	17 (18.7)
Total	91

[a] Frequencies were calculated excluding tumors with information not available for specific variables because of lack of tissue samples; two patients with bilateral BC (out of the three identified) were considered separately for each tumor

All BRCA1/2 deleterious mutations were detected in the 65 cases with an a priori probability above 10% (expected versus observed: 36.4 and 15.4%, respectively) and none in

Table 2 Personal and family history of cancer, pathologic characteristics and immunohistochemical staining for ER, PR, HER2 and MIB1 in the *BRCA1/2* MBC mutation carriers (Florence, Italy 1990–2007)

Gene	Mutations[a]	Mutation effect	Case code	Personal history of breast cancer (age at diagnosis, year)	Breast/ovarian cancer in first-degree relatives (age at diagnosis, year)	Histology/ Grade	Immunostaining			
							ER	PR	HER2	MIB1
BRCA1	3347delAG	R1076fs1084X	MB 5[b]	60	2 Breast (45, 41)	IDC/3	+	+	−	−
	3347delAG	R1076fs1084X	MB 56	55		ILC	−	−	−	+
BRCA2	**802insA**	M192fs205X	MB 83	70		IDC/2	+	+	−	na
	IVS7-2A>G	Exon 8 skipping	MB 115	41		IDC/3	+	−	+	−
	1003delA	R259fs276X	MB 16[b]	64		Papillary/3	−	−	+	+
	2041insA	I605fs615X	MB 103	44	Breast (54)	IDC/3	−	−	+	−
	5301insA	W1691fs1694X	MB 90	57		IDC/2	+	−	−	+
	6010G>T	E1928X	MB 31[b]	53	3 Breast (48, 45, na)	Paget's/3	−	−	+	+
	6696delTC	S2156fs2174X	MB 3[b]	63	Ovary (62)	IDC/3	+	+	+	−
	7935delT	G2569fs2647X	MB 89[c]	74–77	Breast (41), ovary (58)	IDC/3	+	+	na	na

IDC invasive ductal carcinoma, *ILC* invasive lobular carcinoma; *na* not available

[a] Novel mutations are reported in bold; [b] cases reported in our previous study [25]; [c] bilateral BC
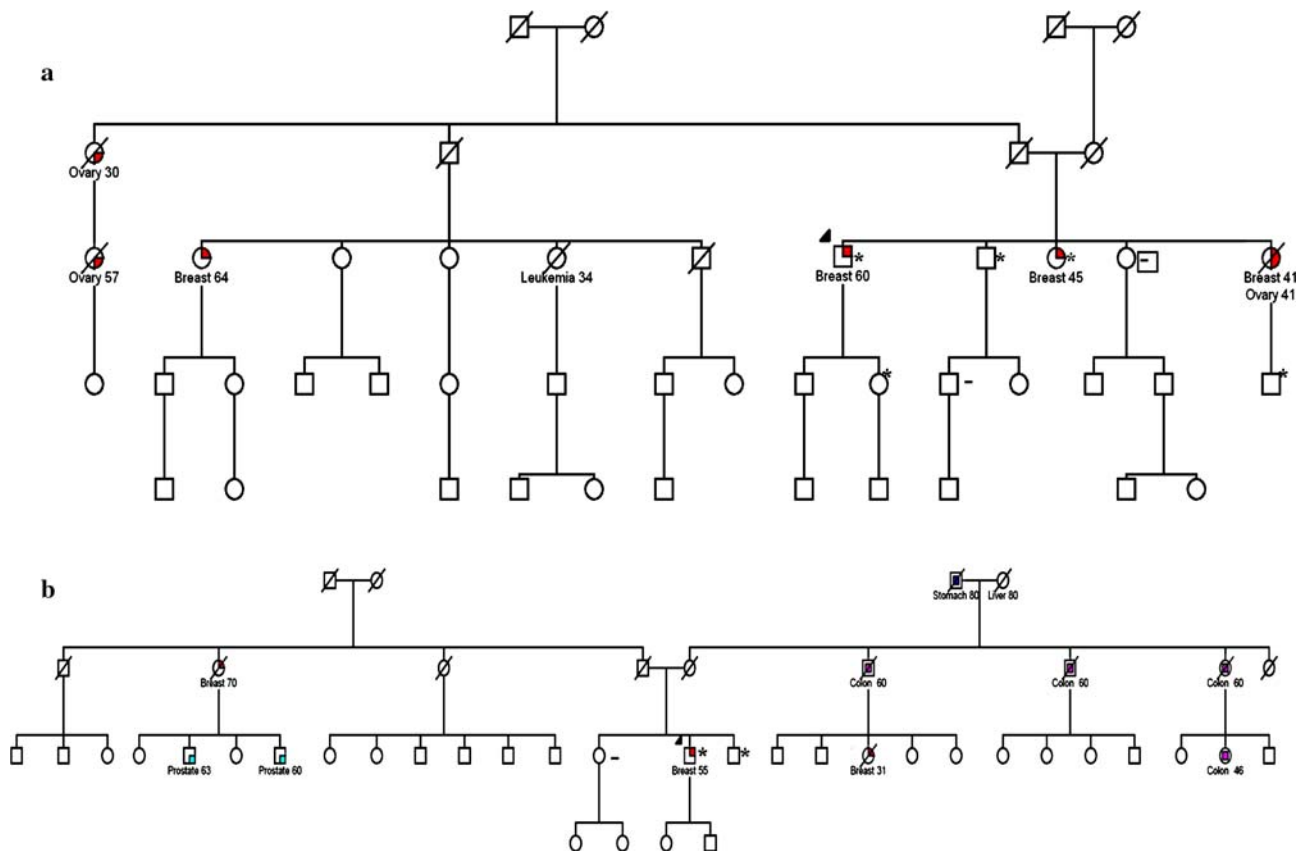

Fig. 1 Pedigrees of the two families carrying the *BRCA1* 3347delAG germ-line mutation. Probands MB5 (**a**) and MB56 (**b**), are indicated by *arrows*; *: Mutation carrier; −: non carrier

the 43 cases with an a priori probability below 10% (expected versus observed: 4.9 and 0%, respectively). In particular, we identified *BRCA1/2* mutations in 3 of the 6 MBC patients with an a priori probability above 80%

(expected versus observed: 95.3 and 50.0%, respectively). Indeed, one MBC case with a BRCAPRO 4.2 of 100% was shown to carry a mutation in *p53*, the main gene associated with Li-Fraumeni syndrome (data not shown).
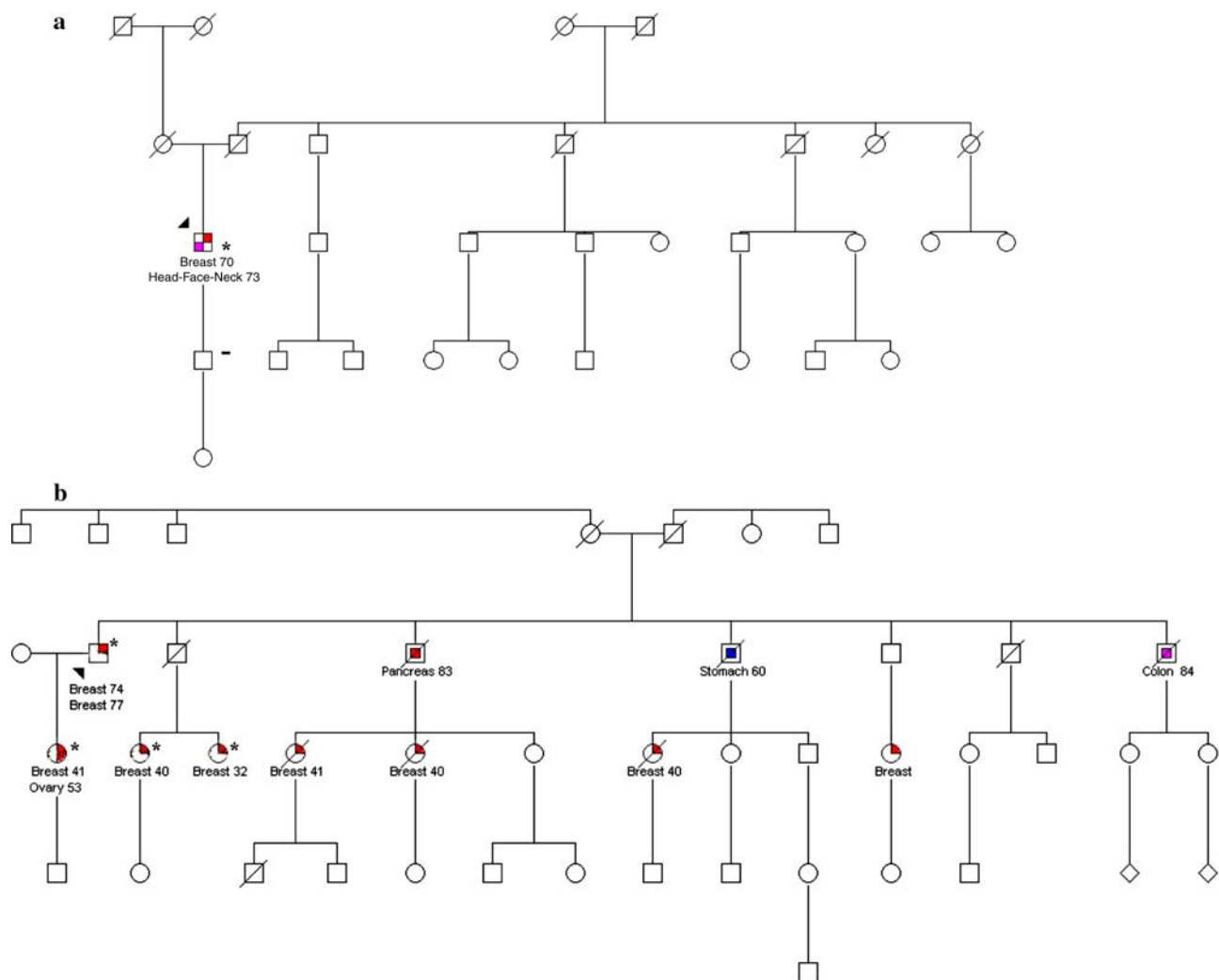
Fig. 2 Pedigrees of families carrying two novel *BRCA2* germ-line mutations: *BRCA2* 802insA (**a**) and *BRCA2* 7935delT (**b**). Probands, MB83 (**a**) and MB89 (**b**), are indicated by arrows. *: Mutation carrier; –: non carrier

Clinical-pathologic characteristics and associations with *BRCA1* and *BRCA2* mutation status

To further characterize *BRCA1/2*-related MBCs we analyzed the phenotypic features of tumors and investigated the associations between clinical-pathologic characteristics and mutation status. The median age at BC diagnosis among *BRCA1/2* mutation carriers tended to be lower than that among cases in which mutations were not detected (median: 58.5 years; range: 41–77 years, vs. median: 66.3 years; range: 24–90 years). With regard to first-degree FH of breast/ovarian cancer, 5 of the 10 (50.0%) *BRCA1/2* mutation carriers reported a positive FH. Overall, 5 out of 28 (17.9%) MBCs with a positive FH and 5 out of 80 (6.3%) MBCs with a negative FH carried *BRCA1/2* mutations, with a threefold association emerging between a positive FH and carrier status: the point estimate of the prevalence rate ratio was 2.86, falling short of

the level of statistical significance (95% CI, 0.89–9.14). With regard to pathologic characteristics, of the two *BRCA1*-tumors, one was an invasive ductal carcinoma, ER+, PR+, HER2− and MIB1−, and the other was a lobular carcinoma, ER−, PR−, HER2− and MIB1+ (Table 2). Overall, of the two *BRCA1*-tumors one resulted with a triple negative (ER−, PR−, HER2−) phenotype. The majority of the *BRCA2*-tumors were invasive ductal carcinomas (75%, 6/8) and were of high grade (75%, 6/8), 62.5% (5/8) were ER+, 62.5% (5/8) were PR−, 50% (3/6) were MIB+ and 71.4% (5/7) were HER2+ (Table 2). We further investigated whether *BRCA2*-related MBCs showed specific phenotypic profiles by analyzing associations between *BRCA2* mutation status and pathological characteristics (Table 3). Statistically significant associations emerged between *BRCA2* mutations and absence of PR expression ($P = 0.008$), positive HER2 immunostaining ($P = 0.002$) and high tumor grade ($P = 0.005$). No

Fig. 3 Transcript characterization of the *BRCA2* IVS7-2G>A variant. **a** RT-PCR products, visualized on ethidium-stained agarose gel, showed a smaller cDNA fragment (*arrow*) from RNA of the patient carrying the *BRCA2* IVS7-2G>A variant compared to control cDNAs; **b** sequencing analysis of the abnormal PCR product showed loss of exon 8; **c** graphic model of the effect of *BRCA2* IVS7-2G>A variant
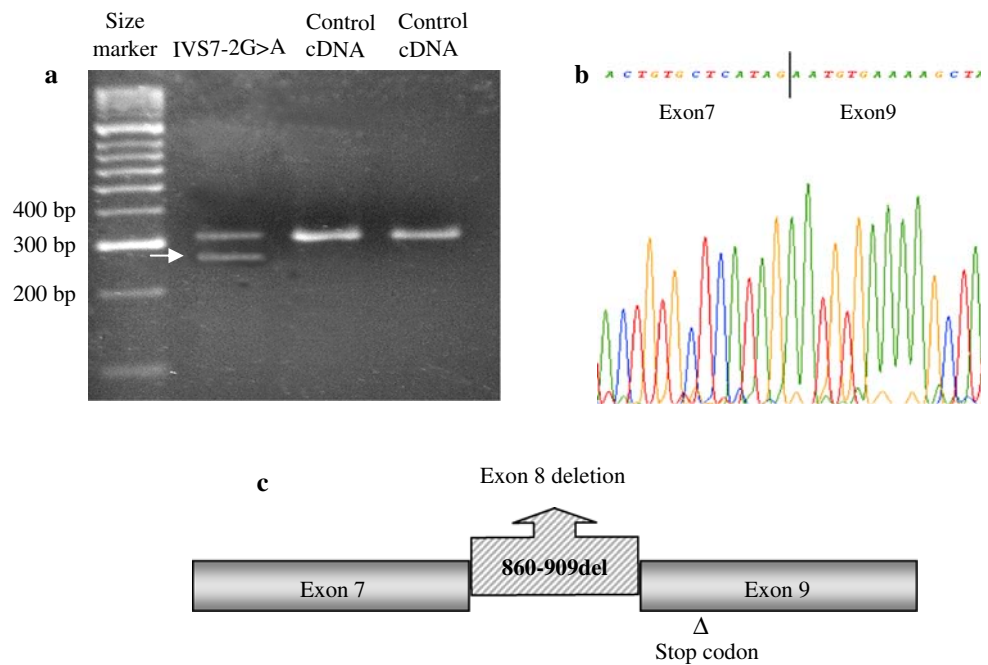

Table 3 Association between selected pathological characteristics and *BRCA2* mutation status in MBCs (Florence, Italy 1990–2007)

Parameter	*BRCA1/2* wild type[a] N (%)	*BRCA2* mutation N (%)	P value*
Grade			
G1 + 2	66 (76.7)	2 (25.0)	0.005
G3	20 (23.3)	6 (75.0)	
Total	86	8	
ER			
Negative	8 (9.3)	3 (37.5)	0.05
Positive	78 (90.7)	5 (62.5)	
Total	86	8	
PR			
Negative	14 (16.3)	5 (62.5)	0.008
Positive	72 (83.7)	3 (37.5)	
Total	86	8	
MIB1			
Negative	54 (68.4)	3 (50.0)	0.39
Positive	25 (31.6)	3 (50.0)	
Total	79	6	
HER2			
Negative	70 (85.4)	2 (28.6)	0.002
Positive	12 (14.6)	5 (71.4)	
Total	82	7	

* Fisher exact test

[a] The 2 *BRCA1* mutation carriers were excluded from this analysis

statistically significant association was found between *BRCA2* mutations and other clinical-pathological characteristics analyzed, including ER and MIB1 expression. Overall, a specific phenotypic profile, characterized by PR−, HER2+ and high tumor grade, emerged for *BRCA2*-related MBCs.

Discussion

Here, we report the results of a large population-based study of 108 Italian MBC cases, from Tuscany (Central Italy), performed to investigate the prevalence and spectrum of *BRCA1/2* germ-line mutations and to characterize

the phenotypic features of *BRCA1/2*-related and unrelated MBCs.

Overall, the *BRCA1/2* mutations prevalence (9.3%) observed in our population-based series is comparable with those reported in MBCs unselected by FH from other different populations [23, 28–30]. Although *BRCA2* mutations are currently considered as the major genetic risk factor for MBC, the association between MBC and *BRCA1* mutations is not as well established [3]. *BRCA1* mutations are quite rare in unselected MBC cases being more frequent in specific populations in which a founder effect is known to occur [6]. Notably, the same *BRCA1* mutation (3347delAG), represented the overall *BRCA1* mutation frequency (1.9%) in the present study. We recently reported that the *BRCA1* 3347delAG is a founder mutation in Tuscany [31], thus confirming that unselected series of cases from specific geographic areas may lead to the identification of *BRCA1/2* mutation with founder effect in genetically heterogeneous countries, such as Italy.

Of the 10 mutations identified in our MBC series, three, including two *BRCA2* frameshift mutations (802insA and 7935delT) and a *BRCA2* intronic variant (IVS7-2 A>G), were novel alterations not reported in the BIC database (http://research.nghgri.nih.gov/bic). We demonstrated, by characterizing the transcript produced from RNA of the MBC patient carrying the variant, that the *BRCA2* IVS7-2 A>G is a novel splice site deleterious mutation causing loss of *BRCA2* exon 8 and creating a premature stop codon. In this respect, it is noteworthy that about 4% of the genetic variants in the BIC database are reported as splice site alterations. Overall, in the present study, we enlarged the mutational spectrum of *BRCA2* gene and provided further data to contribute to the discussion on the causal role and clinical relevance of intronic variants.

As we recently observed in FBC [32], when considering the frequency of mutations in MBC cases stratified according to BRCAPRO 4.2, the prediction accuracy improves as the threshold probability increases. Indeed, we found that 66.6% of MBC patients with probability prediction ≥80% have mutations in the *BRCA1/2* genes and in other genes, such as *p53*. However, a degree of over-estimation is evident. Despite the comprehensive scan of the coding sequence and the intron-exon boundaries there remains a substantial chance of missed *BRCA1/2* genetic alterations possibly due to mutations in other regions of the genes or due to genomic rearrangements. In this respect, it is noteworthy that we recently shown that no *BRCA1/2* rearrangements were found in a large series of Italian MBCs, including 72 mutation-negative MBC cases from the population-based series here analyzed [17]. Thus, the data reported in the present study reflect the overall population prevalence of *BRCA1/2* mutations in Italian MBC cases. Given the reasonably high sensitivity of our screening and the sample size, our results indicate that, at population level, a large fraction of MBC cases are not accounted for by *BRCA1/2* alterations. Collaborative studies are essential to better investigate the pathogenesis of MBC, and, in this context, MBC cases in our series may be useful in identifying new genes predisposing to moderate/high MBC risk. From a clinical point of view, our data suggest that probands with a high (≥80%) carrier probability prediction have to be considered as carriers of a strongly predisposing genetic alteration either in *BRCA1/2* genes or in other yet unknown BC predisposing genes, independently of our ability to detect such mutations.

Consistent with published data on MBC from diverse populations [3, 23, 28], in our population-based series, the mean age of BC presentation in males was 63 years and about one-fourth of MBC cases reported a first-degree BC-FH, thus indicating that our series is representative of a standard MBC population and suggesting that data obtained analyzing the present series can be relevant for MBC in general. We observed that the median age at BC diagnosis among *BRCA1/2* mutation carriers tended to be younger (median: 58.5 years) than that of negative cases (median: 66.3 years). *BRCA1/2* mutations were more prevalent in men with a positive first-degree FH (17.9%) compared with those without (6.3%) and a threefold association emerged between a positive FH and carrier status, falling short of the level of statistical significance. It has been previously reported that *BRCA1/2* mutations are more frequent in MBCs with a positive FH [23, 29, 33]. However, in our series, 50% of the mutations were identified in MBC cases without FH, thus from a clinical point of view, our data indicate that mutation screening is beneficial also among isolated MBC cases.

With regard to pathological characteristics of the tumors, in our series the majority of breast tumors were invasive ductal carcinomas and express ER/PR, whereas only a minority over-expresses HER2. Overall, our results are consistent with retrospective studies on large MBC series performed to characterize the major phenotypic features of BC in males (18, 21). To further investigate the pathological features of BC in men, we also examined the proliferative activity of MBCs by immunohistochemical evaluation of MIB1. Proliferative activity is considered an important prognostic factor in FBC, but it has been scarcely investigated in MBC and data are often inconsistent, most likely because of the small number of cases examined [34, 35]. Our results showed that about 34% of MBCs exhibit a high proliferative activity (MIB1+). At present, little is known of the immunophenotypic characteristics of MBCs stratified according to *BRCA1/2* mutation status [21]. Notably, one of the two *BRCA1*-related MBC, identified in our series, showed a triple negative phenotype (ER−, PR−, HER2−) consistent with what observed in *BRCA1*-related breast

tumors in females [19]. Furthermore, we observed that *BRCA2*-related MBCs showed a significant association with HER2 over-expression and with a high tumor grade, thus confirming on a large number of cases what we have previously reported [25]. Intriguingly, we found that *BRCA2*-related MBC tended to be negative for PR expression. Taking into account that MBCs are predominantly PR+ and HER2−, our findings suggest that specific phenotypic characteristics are associated with *BRCA2*-linked MBCs. These findings may be relevant on a clinical ground considering that a tumor phenotype characterized by PR−, HER2+ and high grade may be indicative of aggressive behavior. Furthermore, our results may be also relevant for the treatment of MBC, by providing data on biomarkers useful to determine the subset of MBC patients (PR−, HER2+) that will benefit from trastuzumab therapy. Overall, our observations may have important prognostic and therapeutic implications and warrant to be replicated in larger collaborative investigations of this rare disease.

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