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EPIDEMIOLOGY

BRCA1/BRCA2 rearrangements and *CHEK2* common mutations are infrequent in Italian male breast cancer cases

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Abstract Male breast cancer (MBC) is a rare and poorly known disease. Germ-line mutations of *BRCA2* and, to lesser extent, *BRCA1* genes are the highest risk factors associated with MBC. Interestingly, *BRCA2* germ-line rearrangements have been described in high-risk breast/ovarian cancer families which included at least one MBC case. Germ-line mutations of *CHEK2* gene have been also implicated in inherited MBC predisposition. The *CHEK2* 1100delC mutation has been shown to increase the risk of breast cancer in men lacking *BRCA1/BRCA*2 mutations. Intriguingly, two other *CHEK2* mutations (IVS2+1G>A and I157T) and a *CHEK2* large genomic deletion (del9-10) have been associated with an elevated risk for prostate cancer. Here, we investigated the contribution of *BRCA1*, *BRCA2* and *CHEK2* alterations to MBC predisposition in Italy by analysing a large series of MBC cases, unselected for breast cancer family history and all negative for *BRCA1/BRCA2* germ-line mutations. A total of 102 unrelated Italian MBC cases were screened for deletions/duplications of *BRCA1*, *BRCA2* and *CHEK2* by multiplex ligation-dependent probe amplification. No *BRCA1*, *BRCA2* and *CHEK2* genomic rearrangements, including the *CHEK2* del9-10, were found in the series analysed. Furthermore, none of the MBC cases and 263 male population controls, also included in this study, carried the *CHEK2* 1100delC, IVS2+1G>A and I157T common mutations. Overall, our data suggest that screening of *BRCA1/2* rearrangements is not advantageous in MBC cases not belonging to high-risk breast cancer families and that common *CHEK2* mutations play an irrelevant role in MBC predisposition in Italy.

Keywords Male breast cancer · *BRCA1* · *BRCA2* · *CHEK2* · Germ-line mutations · Large genomic rearrangements · MLPA

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Introduction

Male breast cancer (MBC) is a rare and still poorly known disease compared to breast cancer (BC) in females [1]. In Italy, it accounts for 0.2% of all cancers in males and incidence rates, standardised on European population, are approximately 1 new case × 100,000 male residents per year [2]. A positive BC family history (FH) is associated with increased MBC relative risk and about 20% of MBC patients have a first-degree relative with the disease [3]. MBC predisposition can result from germ-line mutations in *BRCA2* (OMIM #6600185) and, at lower extent, in *BRCA1* (OMIM #113705) genes. The frequency of *BRCA1/BRCA2*

mutations ranges from 4 to 40% for *BRCA2* and up to 4% for *BRCA1*, being higher in the presence of founder effects [3–5]. Although *BRCA1/2* mutations are more frequent among MBC cases with a positive BC-FH, they have also been reported among FH-negative MBC patients [4, 6], thus indicating that mutation screening is beneficial also among MBC cases with no FH.

In addition to point mutations, *BRCA1* and *BRCA2* genes are also affected by large genomic rearrangements. In the last few years, *BRCA1/BRCA2* germ-line rearrangements have been extensively studied in high-risk breast/ovarian cancer families [7, reviewed in 8, 9–19]. In this familial setting the frequency of *BRCA1* rearrangements results higher compared to that observed for *BRCA2* and the majority of *BRCA2* rearrangements are identified in high-risk families that included at least one MBC case [10, 12, 14, 19]. Thus, 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* rearrangements in high-risk families. Germ-line *BRCA2* rearrangements were observed in 7 to 13% of MBC families from different populations, including French, Australian, Spanish and Portuguese [10, 14, 19, 20]. However, no *BRCA1/BRCA2* rearrangements were found in MBC families of German origin [16] and no *BRCA2* rearrangements were identified among Finnish MBC cases unselected for BC-FH [21]. Difference in genetic background and in case selection criteria, and the relatively small number of MBC cases, thus far analysed, could explain discrepancies in *BRCA1/2* large genomic rearrangements detection rate observed in various studies. Considering that the overall frequency of *BRCA2* rearrangements is rare, the relevance of MBC is an important issue.

There is some evidence implicating the low-penetrance BC susceptibility gene *CHEK2* (OMIM #604373) in inherited MBC predisposition. In particular, the *CHEK2* 1100delC mutation has been shown to confer approximately a 10-fold increase of BC risk in men lacking *BRCA1/BRCA2* mutations and it was estimated to account for 9% of MBC cases [22]. Although this mutation has been strongly associated with the increased MBC risk in high-risk BC families this association is not so evident in series of MBC cases unselected for FH [23–25]. Interestingly, the contribution of the *CHEK2* 1100delC mutation to BC predisposition varies by ethnic group and from country to country. A decreased frequency of the 1100delC allele in North to South orientation has been observed in Europe [26, 27]. In Italy, this variant has been shown to play an irrelevant role for BC risk in female [28], however, the role of the *CHEK2* 1100delC has not been investigated in Italian MBC. Two other common *CHEK2* mutations, the IVS2+1G>A and the I157T, have been associated with an elevated risk for BC and prostate cancer [29–32]. The contribution of these *CHEK2* mutations to BC susceptibility is still debated in females and no data are available in males [33, 34]. Furthermore, a *CHEK2* large genomic deletion (del9-10), leading to loss of exons 9 and 10, has been recently identified as a founder mutation in Czech, Slovak and Polish populations and has been associated with an increased risk of BC and prostate cancer [12, 35, 36]. No data are currently available to what extent this deletion is responsible for cancer burden in other populations.

In the present study, we evaluated the contribution of *BRCA1*, *BRCA2* and *CHEK2* large genomic rearrangements in inheritance of MBC predisposition in Italy by assessing their prevalence in a large series of MBC cases unselected for BC-FH and all negative for *BRCA1* and *BRCA2* point mutations. A total of 102 unrelated Italian MBC cases were included in this study and screened by Multiplex Ligation-dependent Probe Amplification (MLPA). To further investigate the role of *CHEK2* in MBC susceptibility, we also analysed the prevalence of the three common *CHEK2* mutations, the 1100delC, IVS2+1G>A and I157T, in all MBC cases and in 263 healthy adult male population controls included in this study.

Patients and methods

A total of 102 unrelated Italian MBC cases, all *BRCA1/BRCA2* mutation negative, were included in this study irrespectively of breast/ovarian cancer FH. MBC cases were identified at four centres in Italy: the *CSPO*-Scientific Institute of Tuscany (Florence), the *Cancer Genetic Counselling Center*, Department of Experimental Medicine of the University "La Sapienza" (Rome), the *Regional Reference Centre for the Biomolecular Characterization and Genetic Screening of Hereditary Tumors*, University of Palermo (Palermo) and the *Clinical Experimental Oncology Laboratory*, National Cancer Institute (Bari). All MBC patients signed informed consent form with description of the study protocol, including the information about the mutational analysis of the *BRCA1/BRCA2* genes. Overall, our conventional screening approaches included the analysis of the full coding sequence and intron/exon boundaries of both *BRCA1* and *BRCA2* genes by combining PTT, SSCP and direct sequencing [6, 18, 37]. For each study participant we obtained information on his FH for cancer at any sites, including all first- and second-degree relatives. Procedures to maintain confidentiality for all the information collected were strictly applied. A series of 263 healthy adults males were also included in this study as representative of control population. All participants signed an informed consent form and provided a blood sample. The study was approved by local ethical committees. Genomic

DNA of MBC patients and population controls was extracted from peripheral blood lymphocytes by means of standard phenol-chloroform extraction.

MLPA analysis

BRCA1, *BRCA2* and *CHEK2* deletions/duplications were investigated by Multiplex Ligation-dependent Probe Amplification (MLPA) using the SALSA MLPA KIT P002 BRCA1, which includes probes for each of the 24 exons of *BRCA1*, and the SALSA MLPA KIT P045 BRCA2/CHEK2, which includes probes for *BRCA2* exons 1–4, 7–22, 24, 25, 27 and probes for promoter region located about 2kb before *CHEK2* exon 1 and for *CHEK2* exon 9 (MCR Holland, Amsterdam, The Netherlands). MLPA probes were hybridised to target sequences, ligated and amplified in a PCR reaction using the GeneAmp PCR System 9700 (Applied Biosystems, Warrington, UK) thermal cycler system, as previously described [18]. Briefly, denatured genomic DNA was hybridised overnight with the MLPA probes, PCR amplification of the ligation products was carried out with FAM-labelled primers using the alternative PCR protocol 2. Each PCR product, diluted in GeneScan-Rox 500 size standards and deionized formamide, was run on an ABI 3100 Genetic Analyzer (Applied Biosystem, Warrington, UK). Fragment analysis took advantage of the Genescan 3.1 software (Applied Biosystems, Foster City, CA, USA). For the statistical analysis we transferred the size and the peak areas of each sample to an Excel file and the peak areas of expected MLPA products were evaluated by comparison with a normal control and by cumulative comparison of all samples within the same experiment. DNA samples showing probes with a dosage value less than 0.7 or greater than 1.2 were tested again. For quality control, samples with known *BRCA1/BRCA2* rearrangements, kindly provided by Dr. Marco Montagna, were included in every MLPA reaction. Putative *BRCA1/2* rearrangements were analysed by performing reverse transcriptase PCR (RT-PCR) and Real-Time quantitative PCR (qPCR) approaches. RT-PCR analysis of the *BRCA1/BRCA2* transcript was carried out using Superscript II (Invitrogen, Carlsbad, CA, USA) reverse transcriptase with gene-specific primers for the cDNA. Total RNA extractions were carried out by using Trizol reagent from peripheral blood leukocytes, as recommended by the manufacturer (Invitrogen, Carlsbad, CA, USA). SYBR green Real-Time qPCR was performed on a 7900 Real Time thermocycler (Applied Biosystem, Warrington, UK), as previously described [18]. Briefly, dilutions of a control DNA were used to generate calibration curves for each exon and, for each sample, the values obtained for each *BRCA1/BRCA2* exon investigated were normalized on reference exon values. To exclude single base changes impairing the ligation reaction, the MLPA probe ligation sites were analysed by direct sequencing. Sequencing reactions were performed using an ABI PRISM DyeDeoxy Terminator Cycle Sequencing Kit and ABI 3100 Genetic Analyzer (Applied Biosystems, Warrington, UK).

CHEK2 mutations analysis

The presence of the three common *CHEK2* mutations, the 1100delC, IVS2+1G>A and I157T, was investigated in all MBC cases and controls. MBC cases were screened for the *CHEK2* 1100delC by using the SALSA MLPA KIT P045 BRCA2/CHEK2 which includes a specific probe for *CHEK2* exon 10 resulting in a 490 bp amplification product in the presence of this mutation. Direct sequencing of *CHEK2* exon 10 was performed to screen controls and to verify the MLPA results in a half of the MBCs (56 cases) analysed. Because of the presence of several *CHEK2*-related pseudogenes in the human genome, two primers sets were specifically designed for nested-PCR. The *CHEK2* IVS2+1G>A and I157T (470 T>C) mutations, located in intron 2 and exon 3, respectively, were analysed by PCR-RFLP. A genomic region covering both *CHEK2* intron 2 and exon 3, was amplified by PCR using mutagenic primers to allow for a subsequent restriction enzyme screening. The 194 bp amplification product is cleaved by *Pst*I (New England Biolabs, Beverly, MA) in the presence of the I157T mutation, whereas for the IVS2+1G>A the wild-type product is cleaved by *Scr*FI (New England Biolabs, Beverly, MA). Primers sequences and amplification conditions are available upon request.

Results

In order to evaluate the prevalence and the spectrum of *BRCA1* and *BRCA2* genomic rearrangements in MBC, we screened a series of 102 unrelated Italian MBC cases with no detectable *BRCA1/BRCA2* point mutations, by MLPA. All MBC patients were included irrespectively of breast/ovarian cancer FH. Among all cases, a positive FH of breast-ovarian cancer in at least one first-degree relative was reported in 25 of the 102 (24.5%) MBC patients (Table 1). In particular, 10 (10/102, 9.8%) MBC cases belonged to high-risk breast-ovarian cancer families with two or more BC cases or additional high-risk features such as early age at BC diagnosis (less than 40 years) or bilateral BC (Table 1). Notably, one case belonged to MBC family in which another MBC case was diagnosed at 56 years of age (Table 1). Overall, age at BC diagnosis ranged between 24 and 90 years (median, 67 years). All 102 MBC

Table 1 Distribution of the 102 unselected MBC cases analysed according to breast/ovarian cancer family history in first-degree relatives

	No. of subjects (%)
Total MBC patients	102
MBC cases with negative FH	77 (75.5%)
MBC cases with positive FH	25 (24.5%)
MBC cases with high-risk FH	
Breast/Ovarian Cancer in 2 or more relatives	3
Early onset BC (<40 years)	4
Bilateral BC	2
MBC	1
Total	10 (9.8%)

cases analysed resulted negative for *BRCA1* rearrangements. One sample showed an abnormal *BRCA2* MLPA profile. Based on the reduced probes dosage (a dosage quotient less than 0.6) a *BRCA2* exon 10 deletion could be suspected (Fig. 1a). This result was consistently replicated in three independent experiments. To confirm this data by using different techniques, we performed a qPCR assay that we recently developed and successfully applied for quantifying specific *BRCA1* exons dosage [18]. Here, the dosage of *BRCA2* exon 10 region, normalized on reference *BRCA2* exon 25 values, was performed by means of qPCR and no exon 10 loss was detected (data not shown). To exclude the presence of sequence variants that could affect the MLPA reaction, direct sequencing of a 139 bp PCR fragment, encompassing the ligation site (nt 1376) of the MLPA probe for *BRCA2* exon 10, was performed. An heterozygous A>T substitution at nt 1374, was identified in the genomic DNA of the MBC case showing the altered MLPA profile. This *BRCA2* variant, 1374 A>T (K382N), is not reported in the BIC database (http://research.nhgri.nih.gov/bic). Since it is located just 1 bp upstream the ligation site, it may potentially affect the ligation reaction at the exon 10 probe binding site and cause the altered MLPA profile (Fig. 1b). Overall, no *BRCA2* rearrangements were found in the 102 MBC cases analysed. In this study, we used the SALSA MLPA KIT P045 BRCA2/

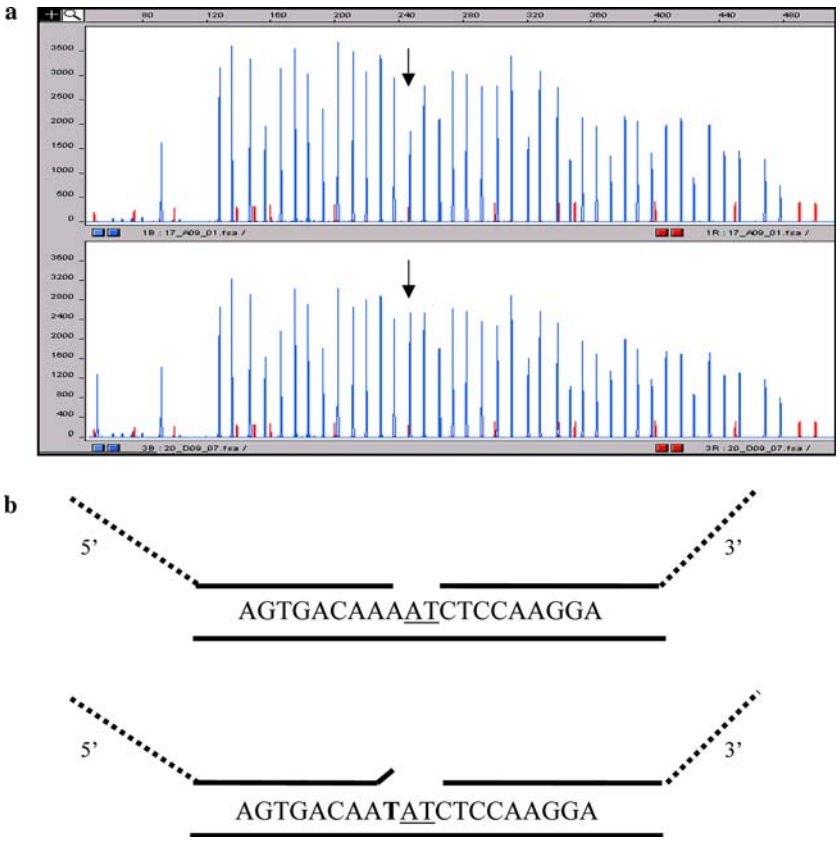

Fig. 1 (**a**) Electropherograms of *BRCA2* MLPA analysis. Comparison between a MBC sample showing *BRCA2* exon 10 signal reduction (top) and a control sample (bottom). Arrows indicate the *BRCA2* exon 10 probe position. (**b**) Graphic explicative model of *BRCA2* exon 10 signal reduction. The presence of the heterozygous germ-line *BRCA2* 1374 A>T substitution, occurring near to the exon 10 probe binding site (underlined), creates a mismatch (bold) and affects the ligation reaction, thus causing a reduction of *BRCA2* exon10 probe signal

CHEK2 (MCR Holland, Amsterdam, The Netherlands). Since this kit contains a control probe specific for the *CHEK2* 1100delC mutation, it allows the screening of this mutation simultaneously in the same MLPA experiments. Thus, we examined the presence of the *CHEK2* 1100delC mutation in all 102 MBC cases analysed by MLPA. Moreover, 263 healthy adult male population controls were screened for this mutation by direct sequencing. None of the MBC cases and controls carried the *CHEK2* 1100delC mutation. To further analyse the role of *CHEK2* alterations in MBC predisposition, we extended our screening to include two other common *CHEK2* mutations, the IVS2+1G>A and the I157T (470 T>C). All 102 MBC cases and 263 controls were genotyped for the presence of these two mutations by PCR-RFLP. None of the MBC cases or controls carried the *CHEK2* IVS2+1G>A and I157T. Finally, taking advantage of using the SALSA MLPA KIT P045 BRCA2/CHEK2 we could also exclude the presence of *CHEK2* rearrangements, including the *CHEK2* del9-10, in all 102 MBC cases tested by MLPA.

Discussion

In this paper, we report the results of the first multi-centre study performed to investigate the prevalence and the spectrum of *BRCA1* and *BRCA2* genomic rearrangements in Italian MBC cases. A large series of MBC patients, unselected for breast/ovarian cancer FH and with no detectable *BRCA1/BRCA2* germ-line point mutations, was analysed by MLPA. No *BRCA1* and *BRCA2* genomic rearrangements were detected in the 102 MBC cases analysed. Currently *BRCA1/BRCA2* germ-line rearrangements are investigated in high-risk breast/ovarian cancer families and a higher frequency of *BRCA1*, compared to *BRCA2*, rearrangements is reported in this familial setting [7–18]. Interestingly, *BRCA2* rearrangements seem to be clustered to high-risk families with at least one MBC case [14, 19, 20], thus indicating the relevance of MBC to select families for *BRCA* genes rearrangements analysis. With regard to MBC, the majority of the studies have analysed relatively small number of MBC cases and have focused on the screening of *BRCA2* rearrangements in familial MBC [10, 14, 19–21]. It is noteworthy that also a *BRCA1* germ-line rearrangement was found in a high-risk BC family that included a case of MBC [12]. Considering that *BRCA1/2* germ-line mutations can be also identified in MBC with no BC-FH [4, 6], in the present study, we wanted to assess the relevance of *BRCA1* and *BRCA2* rearrangements in unselected MBCs by analysing a large series of MBC patients included irrespectively of their breast/ovarian cancer FH. Overall, in our series about 25% of MBC patients reported a positive breast/ovarian cancer FH in at least one first-degree relative and about 10% belonged to high-risk breast-ovarian cancer families. Notably, the fraction of MBC cases with BC-FH in our series was consistent with the overall percentage of FH-positive MBCs reported in the general population [3], thus indicating that our series is representative of a standard MBC population. Taking into account that *BRCA2* rearrangements were found in about 10% of high-risk MBC families [10, 14, 19, 20] and that our series included 10 MBC cases belonging to high-risk families we could have expected to find no more than one case with genomic rearrangements. Overall, our data indicate that *BRCA1/2* rearrangements are irrelevant in the settings of a standard MBC population and suggest that the screening for *BRCA1/2* rearrangements is advantageous only in the context of high-risk MBC families.

Here, we performed MLPA to search for *BRCA1/2* genomic rearrangements. MLPA is a rapid, high sensitive and cost-efficient technique useful to screen large genomic rearrangements [reviewed in 8]. However, MLPA shows some technical limitations since probes target only short sequences in each exon and rearrangements involving other portions could be lost. In our experience false-positive alterations involving single exons were quite common and were resolved by repeated testing or by adopting a multi-step approach implying different PCR-based techniques. In the MBC series screened in this study, a putative *BRCA2* exon 10 deletion was shown to be due to the presence of a novel *BRCA2* sequence variant (*BRCA2* 1374A>T) located at the binding sites for the exon 10 probe. A MLPA profile suggestive of a deletion of the *BRCA2* exon 9 was also observed in a MBC carrier of the *BRCA2* 1003delA germ-line mutation [6] because of the occurrence of this mutation at the ligation site of the MLPA probe for the *BRCA2* exon 9. Thus caution in interpreting data and multiple approaches in validating positive testing results are needed in *BRCA1/2* MLPA screening.

We also investigated the contribution of *CHEK2* mutations on inherited MBC predisposition in Italy by screening MBC cases and population controls for the presence of the three common *CHEK2* mutations (1100delC, IVS2+1G>A and I157T). Since the SALSA MLPA KIT P045 BRCA2/CHEK2, used in this study, contains a control probe specific for the *CHEK2* 1100delC mutation, it allowed us to screen for this mutation all MBC cases in the context of *BRCA2* MLPA analysis. Very recently, the simultaneous screening for the *CHEK2* 1100delC mutation and *BRCA1/2* rearrangements by MLPA has been proposed as a useful strategy in populations in which the *CHEK2* mutation shows a very low frequency [38]. In our study, none of the 102 MBC cases, analysed by MLPA, and none of the 263 healthy adult male population controls, screened by direct sequencing, carried the *CHEK2* 1100delC mutation, thus suggesting that this mutation is very infrequent in the

Italian male population. Indeed, a decreased frequency of the 1100delC allele in North to South orientation has been observed in Europe [26, 27]. In Italy, this variant has been reported to play an irrelevant role for BC risk in females [28]. Here, we showed that the *CHEK2* 1100delC variant does not play a relevant role also for BC risk in males. Although this mutation has been strongly associated with the increased MBC risk in high-risk BC families [22] this association is not so evident in population-based MBC series [23–25]. Our data are concordant to results obtained in other unselected MBC series, including those from Finland, USA, UK and Israel, in which it was reported that the *CHEK2* 1100delC is unlikely to account for a significant proportion of MBC cases [23–25]. To further investigate the role of *CHEK2* in inherited MBC predisposition, we genotyped all 102 MBC cases and 263 controls for two other common *CHEK2* mutations, the IVS2+1G>A and the I157T. None of the MBC cases and controls carried these mutations. The *CHEK2* IVS2+1G>A and I157T were previously associated with an elevated risk for female BC and prostate cancer [29–32], however, this association is still debated [33, 34]. Our results suggest that the *CHEK2* IVS2+1G>A and I157T play an irrelevant role in MBC in Italy. Taking advantage of using the SALSA MLPA KIT P045 BRCA2/CHEK2, which contains a probe specific for *CHEK2* exon 9, we could also exclude the presence of the *CHEK2* del9-10 in all MBC cases screened by MLPA. This large *CHEK2* deletion was recently identified as founder mutation in Czech, Slovak and Polish populations and associated with an increased risk of female BC and prostate cancer [12, 35, 36]. Here, we showed that the *CHEK2* del9-10 does not play a role in MBC predisposition in Italy. It will be of interest to further investigate whether *CHEK2* mutations can be associated with prostate cancer in Italian male population and, on the other hand, to verify whether they can be associated with MBC in other populations. Overall, our data suggest that screening of large *BRCA1/2* rearrangements is not likely to be recommended in MBC cases not belonging to high-risk families as well as screening of common *CHEK2* mutations is not advantageous in Italian MBC cases.

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