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BRCA1 and *BRCA2* Mutation Status and Tumor Characteristics in Male Breast Cancer: A Population-based Study in Italy[1]

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ABSTRACT

To investigate at the population level the impact of *BRCA1/BRCA2* gene alterations in male breast cancer, we analyzed a population-based series of 25 male breast cancer cases from Florence, Central Italy. We combined mutational screening with the study of germ-line allele transcript levels and of tumor-associated losses of heterozygosity. Screening by protein truncation test and single-strand conformational polymorphism assay, followed by sequencing, revealed 4 pathogenetic mutations (4 of 25 = 16%; 95% confidence interval, 5–37%), 1 in *BRCA1* and 3 in *BRCA2,* including mutations recurring in Central Italy (*BRCA1* 3345delAG and *BRCA2* 6696delTC). The *a priori* probability of carrying a mutation, estimated using BRCAPRO software, showed a good agreement between expected and observed mutations (14% *versus* 16%). A 7-fold association between germ-line mutations and family history of breast-ovarian cancer emerged. To investigate associations between *BRCA1/BRCA2* status and clinicopathological characteristics, we analyzed the histopathological and immunophenotypic parameters of the tumors. A significant association emerged between mutation carrier status and high histological grade ($P = 0.02$). Furthermore, one *BRCA2* carrier was affected with Paget's disease, an extremely rare male breast cancer histotype. Overall, *BRCA1/2* mutations were observed to be strongly associated with positive c-erbB-2 immunostaining ($P = 0.004$). To evaluate germ-line allele expression, we used primer extension assays targeting frequent *BRCA1* and *BRCA2* polymorphisms. A *BRCA2* allele transcript imbalance was found in one of four heterozygotes tested, all of them negative for germ-line mutations. *BRCA1* transcript imbalances were not detected in nine heterozygotes analyzed. Losses of heterozygosity at one or more of nine loci in the *BRCA2* region were found in 8 of 22 tumors tested. Interestingly, a case that was negative for *BRCA1/BRCA2* germ-line mutations and that had *a priori* mutation probability <10% showed loss of heterozygosity at all three of the intragenic *BRCA2* markers analyzed, which could be related to a somatic involvement of *BRCA2*. No losses of heterozygosity were detected at *BRCA1*. In conclusion, constitutional *BRCA1/BRCA2* mutations accounted for 16% of the male breast cancer cases in this area of Central Italy. The detection of a *BRCA2* germ-line transcript imbalance and of a somatic loss of *BRCA2* among the cases that resulted negative for germ-line mutations suggests a role of this gene more relevant than indicated by conventional mutational analysis. A distinct pattern of characteristics indicative of aggressive behavior, including high-grade and c-erbB-2 expression, was evident in tumors from germ-line *BRCA2* mutation carriers. This suggests that phenotypic characteristics may contribute to the identification of hereditary *BRCA2*-related male breast cancers and that these tumors might share a unique molecular pathway of cancer progression.

INTRODUCTION

Cancer of the breast is rare in males. In Western countries, in which FBC[3] is the first or second most commonly diagnosed malignancy of women, MBC accounts for less than 1% of all cancers in men. Epidemiological risk factors include pathological conditions associated with primary and secondary hyperestrogenism, radiation exposure, and, particularly, BC FH, which points to a relevant genetic component in disease predisposition (1–2). Furthermore, MBC incidence rates are relatively stable in time, which contrasts with FBC, but tend to vary by race and/or geographical location, suggesting that ethnic and/or locally acting environmental factors may enhance disease risk at the population level (1). Evidence provided by several studies implicate pathogenetic germ-line mutations of *BRCA2* and, with lower frequency, of *BRCA1* in MBC. In fact, mutations in *BRCA1* and *BRCA2* were estimated to be responsible for 16 and 76%, respectively, of the MBCs occurring in high-risk breast/ovarian cancer families (3–5). With regard to MBCs unselected for FH, the prevalence of *BRCA1/BRCA2* mutations has been investigated in population- and clinic-based series from countries with ethnically diverse populations, such as the United Kingdom, Iceland, continental Europe, the United States, and Israel. Mutation frequencies ranged from 4 to 40% for *BRCA2* and up to 4% for *BRCA1*, being higher in populations with founder mutations (5–15). Overall, these studies suggest that migration patterns and genetic bottlenecks could account for regional differences in the contribution of the two genes to disease. However, the frequency of *BRCA1/BRCA2* alterations is probably underestimated in MBCs from populations without highly recurrent founder mutations. In fact, the sensitivity of the most commonly used mutation screening techniques is regarded to be about 70–80% (4). Moreover, extensive genomic deletions, known to account for a subset of *BRCA1*/2-related breast/ovarian cancers, are missed by standard PCR-based screening strategies, whereas mutations in the promoter regions, in regulatory elements and in introns, which interfere with gene expression or with transcript processing, are currently not investigated (16–21). At the somatic level, MBCs arising in *BRCA1/BRCA2* mutation carriers are anticipated to follow the classic double-hit/loss-of-function mechanism, with conversion to homozygosity often attributable to somatic loss of the wild-type allele (5, 22–24).

The phenotypic characteristics of tumors may shed light on the molecular mechanisms implicated in the progression of *BRCA1/BRCA2*-related cancers. In fact, studies conducted on FBCs indicate that *BRCA1*- and, to a lesser extent, *BRCA2*-related tumors tend to manifest specific phenotypic profiles. In particular, BCs arising in female *BRCA1* mutation carriers tend to show high histological grade and high proliferative activity, and to be negative for ER/PR and for

c-erbB-2 (25–31). The genetic and clinicopathological characteristics of MBCs stratified for *BRCA1/BRCA2* status have not been reported.

Population-based series of MBCs from Southern European countries have not been described thus far. We determined the prevalence of pathogenetic *BRCA1/2* mutations in a well-characterized population-based series of MBC patients from Florence, Central Italy. In carriers of frequent *BRCA1* or *BRCA2* polymorphisms, mutational data were complemented with the analysis of germ-line *BRCA1/2* allele expression. Tumors were investigated for LOH at 13q12–14 (*BRCA2*) and 17q21 (*BRCA1*). Finally, *BRCA1/2* status was correlated with cancer FH, tumor histotype, and immunostaining for hormone receptors, c-erbB-2 and Ki-67/MIB1.

PATIENTS AND METHODS

Patients. All of the male patients diagnosed with BC residing in the area of Florence and alive at the end of 1998 were identified and included in an ongoing project. After exclusion of deceased and migrated patients and of those severely ill, eligible subjects were traced and invited to participate in the study. The local Cancer Registry recently estimated the age-adjusted (world standard) incidence rates of BC in males and females in the area to be 0.6 and 67, respectively, per 100,000 residents/year (32). Overall, a series of 31 unrelated MBC patients were contacted and invited to provide a blood sample and detailed personal information; six cases refused, mostly because of advanced age. Twenty-five cases agreed to participate and signed a detailed consent form; all were included irrespective of age, FH, and hospital of original diagnosis and treatment. Histological slides for diagnostic confirmation were obtained for all of the patients, but tissue samples for additional sections were not available for two and scarce for another four cases. A short questionnaire was administered to all of the participants to collect information on the personal and FH of cancer of the breast and/or ovary, and at other sites. All of this information was validated by available sources.

The *a priori* probability of carrying a mutation in *BRCA1* and *BRCA2* was estimated using BRCAPRO software (33, 34). 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 two-tailed Fisher exact test was used for 2 × 2 tables. The study was approved by the Ethical Review Board of the University of Chieti.

Mutational Analysis. The 25 samples were analyzed anonymously. We screened the entire *BRCA1* and *BRCA2* coding sequences, including intron-exon boundaries, using SSCP analysis for *BRCA1* exons 2–10 and 12–24 and *BRCA2* exons 2–9 and 12–27, combined with protein truncation test for *BRCA1* exon 11 and *BRCA2* exons 10 and 11, as described previously (35). Mutations were verified by PCR direct sequencing on two independent blood samples.

Primer Extension Assay. We used a previously described primer extension protocol to quantitate the relative expression of *BRCA1* and *BRCA2* allele transcripts (36). A subset of 16 cases accepted to provide an additional fresh blood sample RNA extraction, which was typed for the frequent polymorphisms *BRCA1* 4427 C→T and *BRCA2* 203 G→A, reported in the BIC database.[4] Heterozygotes were identified after *Ear*I digestion of PCR-amplified *BRCA1* exon 13 (*BRCA1* 4427 C→T), SSCP and PCR-direct sequencing of *BRCA2* exon 2 (*BRCA2* 203 G→A). RNA was isolated using the QIAmp RNA Blood Mini kit, and cDNA was prepared using Sensiscript reverse transcriptase (Qiagen Inc., Chatsworth, CA) in the presence of random hexamers and RNase inhibitor. Primer extension assays were performed as described previously (36) using matched gDNA and cDNA templates, and were confirmed using gDNA and cDNA preparations from independent blood samples. The radioactive signals corresponding to each allele were analyzed using the Molecular Imager System (BIO-RAD, Hercules, CA). Relative transcript expression was estimated by comparing the ratio between the allele signals obtained using cDNA as primer extension template. This ratio was normalized by the corresponding ratio obtained using gDNA as template. A 100% expression was arbitrarily assigned to the allele showing the higher level of expression. The relative expression levels of the allele variants were verified

in repeated assays using two distinct antisense primers. The sequences of the primers used are available on request.

LOH Analysis. Formalin-fixed, paraffin-embedded primary mammary tumor blocks from 23 of 25 cases could be retrieved from the pathology archives of local hospitals, and areas enriched with cancer cells adequate for DNA extraction could be microdissected, as described previously, from dewaxed sections of 22 cases (37). No microdissection could be performed in a case of intraepithelial Paget's disease because tumor cells were not sufficient for the study. Allelotyping of matched blood and tumor genomic DNAs was performed as described previously (37). The markers analyzed[5] were five for *BRCA1* (intragenic: *D17S855, D17S1322, D17S1323*; telomeric: *D17S1183, D17S858*) and 9 for *BRCA2* (flanking: cen-*D13S290, D13S260, D13S171, D13S267, D13S218, D13S263*-tel; intragenic: *D13S1695, D13S1699, D13S1701*). The amplified products were fractionated in a 6% denaturing polyacrylamide gel and visualized by autoradiography. LOH was scored positive when the intensity of alleles varied by more than 50%. Cases with MSI were further tested at the *BAT26* mononucleotide repeat, taken as a sensitive indicator of mismatch repair deficiency (38).

Immunohistochemistry. Immunohistochemical studies were performed on 19 of the 23 cases with available paraffin-embedded blocks. Immunohistochemical data could not be obtained for four patients (including a case of Paget's disease), because tissues were not sufficient for study. Serial sections were mounted on electrostatic slides, air-dried overnight at 37°C, deparaffinized in xylene, and rehydrated in graded alcohols. Endogenous peroxidase activity was blocked in 3% H_2O_2 in methanol for 20 min, after which the slides were washed in PBS (pH 7.4) and treated with normal serum (Lab Vision Corporation, Fremont, CA). The following primary antibodies were applied and incubated overnight at 4°C: anti-c-erbB-2, clone TAB 250 (Zymed, S. Francisco, CA), diluted 1:20; anti-ER, clone 1D5 (BioGenex, San Ramon, CA) diluted 1:30; anti-PR, clone 1A6 (BioGenex, San Ramon, CA), diluted 1:50; and anti-Ki-67, clone MIB1 (Immunoitech, Marseille, France) diluted 1:60. After washing in PBS, the primary antibody was detected by incubation with biotinylated antimouse IgG (Lab Vision), followed by the streptavidin-biotin-peroxidase complex reagent (each for 15 min at room temperature). Immunostaining was visualized with diaminobenzidine-hydrogen peroxidase (Bio-Genex). Sections were lightly counterstained with Mayer's hematoxylin and mounted with Permount (Fisher, Fair Lawn, NJ). Negative control slides were obtained by omitting the primary antibody. For each marker analyzed, the percentages of immunoreactive cancer cells were determined in double-blind fashion by two pathologists (S. B. and V. V.). Discordant cases were discussed, and a consensus statement was reached. According to established criteria, only cancer cells with membrane staining were scored as c-erbB-2-positive (30, 39). Cases were classified as negative or positive for c-erbB-2 when the percentages of immunopositive cancer cells were, respectively, ≤25% or >25%. Slides used for ER, PR, and Ki67/MIB1 staining were scored based on the percentages of positive nuclei (ER/PR positive if >10%; Ki67/MIB1 high if >20%) over the total number of counted cancer cell nuclei (40).

RESULTS AND DISCUSSION

Individual characteristics, mutational data, FH of cancer, histopathology, and immunohistochemical features of the population-based series of 25 MBC cases are shown in Table 1. Age at diagnosis ranged between 42 and 87 years (median, 68 years). The tumors included 18 invasive NOS carcinomas, 1 mixed NOS/colloid carcinoma, 1 mixed NOS/papillary carcinoma, 2 papillary carcinomas, 2 adenoid cystic carcinomas, and, remarkably, a case of Paget's disease of the nipple, associated with intraepithelial neoplasia of the subareolar ducts. Three patients (MB2, MB10, MB22) reported a personal history of metachronous bladder cancers, all of which were diagnosed when they were over 75 years of age. Seven patients (7 of 25, 28%) reported a first-degree FH of breast or ovarian cancer. Thirteen patients (13 of 25, 52%) had a verified first-degree FH of cancer at sites other than breast and ovary, but there was no evidence of specific associations,

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[4] Internet address: http://www.nhgri.nih.gov/Intramural_research/Lab_transfer/Bic/.

[5] Markers analyzed: see Mapview at http://www.gdb.org.

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Table 1 *BRCA1/BRCA2 mutations, personal history and FH of cancer, pathological characteristics, and immunohistochemical staining for ER, PR, c-erbB-2, and Ki67/MIB1 in the series of 25 MBC (MB) cases (Florence, Italy)*

Case	Age at diagnosis (yr)	BRCA1/2 germ-line mutations	Cancers in first-degree relatives		Histology/Grade	Immunostaining			
			Breast/ovary (age at diagnosis, yr)	Other sites (number of affected relatives)		ER	PR	c-erbB-2	MIB1
MB1	69		Breast (33)	Larynx, stomach (3)	NOS/2	+	+	−	−
MB2[a]	78		Breast (63)		NOS/3	+	+	−	−
MB3	63	BRCA2 6696delTC	Ovary (62)	Colorectal	NOS/3	+	+	+	−
MB4	65			Lung	Papillary/1	+	+	−	−
MB5	60	BRCA1 3345delAG	2 × breast (45, 41)		NOS/3	+	+	−	−
MB6[b]	68				NOS/2	+	+	−	−
MB9	50			Lung	NOS/1	+	+	−	−
MB10[a]	83				NOS+colloid/2	+	+	−	−
MB11	42			Melanoma	Papillary/1	na[c]	na	na	na
MB12	58			Brain	NOS/3	+	+	−	+
MB16	64	BRCA2 1003delA		Colorectal	NOS+papillary/3	−	−	+	+
MB18	71				NOS/2	−	−	−	−
MB19	77		Ovary (55), breast (55)		NOS/3	+	+	−	−
MB20	76				NOS/3	na	na	na	na
MB21	87			Larynx	NOS/2	na	na	na	na
MB22[a]	73		2 × breast (60, 48)		NOS/2	+	+	−	−
MB23	71			Lung (2)	NOS/2	+	−	−	+
MB24	70			Colorectal	Adenoid cystic/2	na	na	na	na
MB25	61				Adenoid cystic/3	−	−	−	−
MB26	70				NOS/2	na	na	na	na
MB27	54			Prostate	NOS/1	na	na	na	na
MB28	55	BRCA2 expression imbalance		Lung	NOS/2	+	+	−	−
MB29	71				NOS/3	+	+	−	−
MB30	52				NOS/1	−	−	−	−
MB31	53	BRCA2 6010G→T	3 × breast (48, 45, na)	NHL[d]	Paget's/3	−	−	+	+

[a] Personal history of bladder cancer at age 77, 76, and 78 years, respectively.
[b] LOH at BRCA2 intragenic loci.
[c] na, not available.
[d] NHL, non-Hodgkin's lymphoma.

with the exception of the MB1 family, in which gastric cancer occurred in three first-degree relatives.

Protein truncation test and SSCP screening of the BRCA1/BRCA2 coding sequences and flanking intron-exon borders revealed three frameshift and one nonsense mutations in four cases, one (1 of 25, 4%) in BRCA1 (3345delAG) and three (3 of 25, 12%) in BRCA2 (1003delA, 6010G→T, 6696delTC). All of these mutations are already reported in the BIC database.[4] Interestingly, BRCA2 6696delTC appears to recur in Italy, where it was detected in nine unrelated female BC kindreds (Ref. 35 and unpublished data[6]). Furthermore, BRCA1 3345delAG was independently detected in two unrelated FBC patients from Northeastern Tuscany.[7] In this respect, it is noteworthy that founder BRCA1 and BRCA2 mutations were recently described in Calabria and Sardinia, two Italian regions that show genetic micro-homogeneity (41–42). This suggests that the selection of cases from small geographic areas might lead to the identification of BRCA1/2 founder effects in other Italian regions of ancient settlement, such as Tuscany.

As in other studies, the median age at cancer diagnosis among the cases with mutations tended to be younger than that of the cases in which mutations were not detected (median, 60.0 years; range, 53–64 years, versus median, 66.7 years; range, 42–87 years; Refs. 5–15). Overall, 3 (42.8%) of 7 cases with breast or ovarian cancer FH carried mutations, in comparison with 1 (5.5%) of 18 cases with a negative FH (Table 2). Thus, a 7-fold association emerged between a positive FH and carrier status: the estimate of the prevalence rate ratio was 7.7, but fell short of statistical significance (95% CI, 0.96–62.2). The a priori probability of carrying a mutation was also estimated and compared with the results of mutational analysis, overall showing a good agreement (expected versus observed: 14 and 16%, respectively), as recently reported by other studies (33, 34). The agreement persisted also in the two subgroups of cases classified according to a

10% probability cutoff point, as shown in Fig. 1. Three mutations were detected in the 8 cases with an a priori probability above 10% (expected versus observed: 37.2% and 37.5%), and 1 in the 17 cases with probability below 10% (expected versus observed: 3.1 and 5.9%, respectively).

With regard to histopathology, it is relevant to note that all of the tumors from mutation carriers were of high grade (P = 0.02). The mutation-positive cases included two ductal carcinomas (one in the BRCA1 carrier), a mixed NOS/papillary carcinoma and, remarkably, the case of Paget's disease (Table 1, Fig. 2). This represents the first documented association of this extremely rare MBC histotype (43) with BRCA2. In FBC, the association between mutation carrier status and high tumor grade, already established for BRCA1, is still debated for BRCA2 (28–31). Intriguingly, Paget's disease of the breast is known to be associated with molecular markers of aggressive behavior (44–45). Overall, our data suggest that in MBC a high-grade phenotype is associated with BRCA2 mutations.

There was no consistent association between the spectrum of tumors other than breast and ovary and BRCA1/2 status. In particular, the case with strong FH of gastric cancer and the three cases with metachronous bladder cancer resulted negative for mutations.

Recent reports indicate that alterations in the germ-line expression

Table 2 *Association between first-degree FH[a] of breast/ovarian cancer and BRCA1/2 germ-line mutations in the population-based series of 25 MBC cases (Florence, Italy)[b]*

FH[a]	Germ-line BRCA1/BRCA2 mutation		
	Yes	No	Total
Positive	3	4	7
Negative	1	17	18
Total	4	21	25

[a] At least one first-degree relative with breast or ovarian cancer.
[b] Prevalence rate (P_R) FH: 7/25 = 28% (95% CI, 12–49%); P_R mutations 4/25 = 16% (95% CI, 5–37%); P_R mutations in cases with positive FH: 3/7 = 42.8% (95% CI, 10–82%); P_R mutations in cases with negative FH: 1/18 = 5.55% (95% CI, 0.1–27%); prevalence rate ratio (PRR) = 7.71 (95% CI, 0.96–62.25).

[6] Unpublished data from the Italian Consortium for Hereditary Breast Cancer.
[7] D. Palli, personal communication.

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(▲): cases with *BRCA2* mutation; (●): case with *BRCA1* mutation; (◆): case with imbalance in *BRCA2* allele expression; (■): case with tumor-associated loss of *BRCA2*.
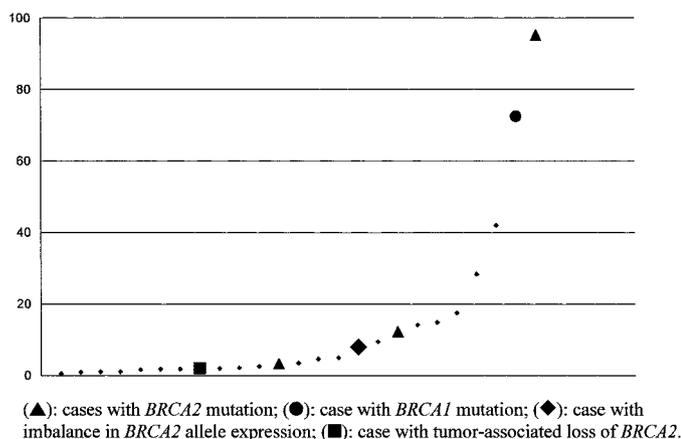
Fig. 1. Distribution of 25 MBC cases ranked according to *a priori* probability of carrying *BRCA1/2* germ-line mutations, as estimated using BRCAPRO (33–34).
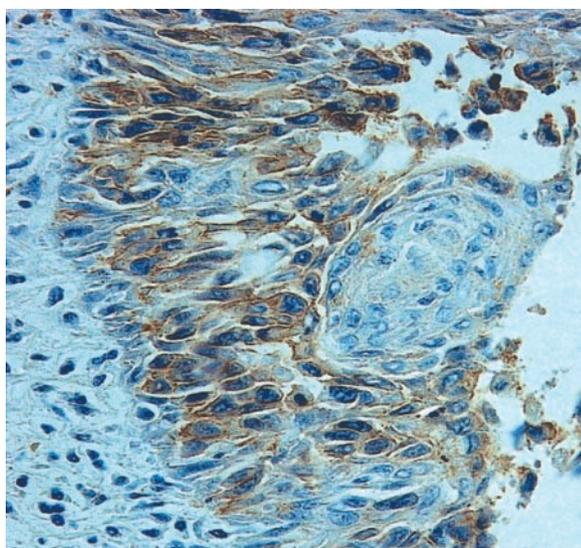

Fig. 2. c-erbB-2 immunohistochemical staining in Paget's disease from case MB31, carrying *BRCA2* 6010G→T. The microscopic field shows intraepithelial malignant cells of a subareolar duct diffusely stained for c-erbB-2 (×400).

of cancer-susceptibility genes may affect predisposition to tumorigenesis (19, 21, 36, 46). We used primer extension assays targeting the frequent polymorphisms *BRCA1* 4427C→T and *BRCA2* 203G→A to evaluate the relative expression levels of *BRCA1* and *BRCA2* allele transcripts in heterozygous cases. Of 16 patients for which RNA was available, 4 resulted heterozygous for *BRCA2* 203G-A and 9 for *BRCA1* 4427C-T. The *BRCA1* carrier resulted heterozygous for *BRCA2* 203G-A and not informative for the *BRCA1* marker; the *BRCA2* carriers were not available for allele expression studies. As shown in Fig. 3, using two distinct antisense primers, an ~5-fold imbalance in the germ-line expression of the *BRCA2* allele bearing T at nucleotide 203 was detected in MB28, one of the four *BRCA2* heterozygotes. This result was confirmed using independent blood samples. Interestingly, MB28 was negative for germ-line mutations, did not report breast/ovarian cancer FH and, as estimated using BRCAPRO (33, 34), had <10% *a priori* probability of carrying a mutation (Fig. 1). No abnormality in *BRCA1* allele expression was detected in the nine patients heterozygous for the *BRCA1* polymorphisms. The presence of a *BRCA2* allele expression imbalance in one of the four cases that could be tested suggests that constitutional transcript deregulation might represent a relevant mode of germ-line

BRCA2 inactivation in MBC. Deregulation of *BRCA1* germ-line expression was not detected in the MBCs analyzed. Thus, the results of the allele expression studies further support the association between *BRCA2* and MBC in the present series of cases.

Previous reports indicate that somatic alterations at loci on chromosomes 13q12, including *BRCA2*, and 17q21, including *BRCA1*, occur quite frequently in MBCs (47). Fig. 4 shows the results of *BRCA1/2* LOH analyses, conducted on 22 of the 25 primary MBCs. No allelic losses at 17q21 were observed in the 19 cases informative at 1 or more of the 5 *BRCA1* polymorphisms (the *BRCA1* carrier was not informative at the loci tested and could not be investigated for loss of the wild-type *BRCA1* allele because of the lack of tumor DNA). These results exclude extensive deletions of *BRCA1* in the tumors studied (Fig. 4). all of the 22 cases were informative at one or more of the nine polymorphisms at or near *BRCA2*. LOH was detected in 8 (36%) of 22 tumors, that included three cases (MB5, MB6, and MB16) with LOH at three or more loci. The tumor from the *BRCA1* carrier MB5 manifested LOH at flanking *BRCA2* markers, but retained the intragenic markers, which suggests that the somatic alterations did not involve *BRCA2*. The tumor from MB6, a case negative for germ-line mutations, showed LOH at flanking and intragenic *BRCA2* markers. When we considered that somatic inactivation of *BRCA2* has been recently reported in sporadic MBCs (48) and that case MB6 had <10% *a priori* probability of carrying a mutation (Fig. 1), we concluded that somatic LOH in this case might be unrelated to germ-line *BRCA2* mutations. The tumor from the *BRCA2* carrier MB16 manifested LOH at loci centromeric and telomeric to *BRCA2* but were not informative at the intragenic loci. The analysis of tumor DNA, conducted using the heterozygous germ-line mutation as an intragenic marker, confirmed the loss of the wild type-allele, in accordance with the classic double-hit mechanism (5, 22–24). The tumor from the other *BRCA2* carrier (MB3) was negative for LOH and could not be further investigated for loss of the wild-type allele because of the lack of DNA. LOH frequencies in informative cases were: *D13S260*, 3 (37.5%) of 8; *D13S267*, 4 (36.3%) of 11; *D13S218*, 2 (33.3%) of 6; *D13S263*, 3 (25.0%) of 12; *D13S171*, 2 (22.2%) of 9; *D13S1699*, 2 (18%) of 11; *D13S1695*, 1 (10%) of 10; *D13S1701*, 1 (10%) of 10; *D13S290*, 0 (0%) of 2. Overall, LOH was most frequent at loci telomeric to *BRCA2*, which could be consistent with literature data, suggesting the presence of MBC-related tumor suppressor gene(s) in this region (47). MSI at 1–5 of the 14 above-mentioned *BRCA1/2* markers (all dinucleotide repeats) was observed in 5 (23%)
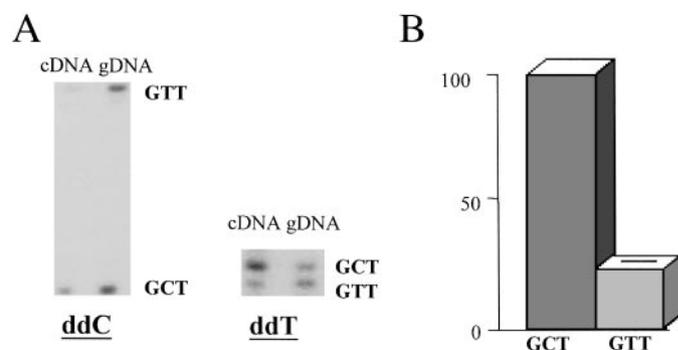

Fig. 3. Analysis of germ-line *BRCA2* allele transcript expression by primer extension in case MB28. The region of *BRCA2* bearing the nucleotide 203 G-A polymorphism was amplified using matched cDNA and gDNA derived from PBLs. These templates were used for primer extension in the presence of ^{32}P-oligonucleotide and the appropriate ddNTP. *A*, primer extension experiments performed using two distinct antisense primers, designed to identify the alleles bearing C or T at nt 203 on the antisense strand respectively by the incorporation of dideoxycytidine (ddC) and of dideoxythymidine (ddT), as indicated. *B*, histogram showing the relative expression levels of the germ-line transcripts from the two alleles. Data are the mean ± SE of two independent determinations, obtained by comparing primer extension signals derived from gDNA and cDNA.
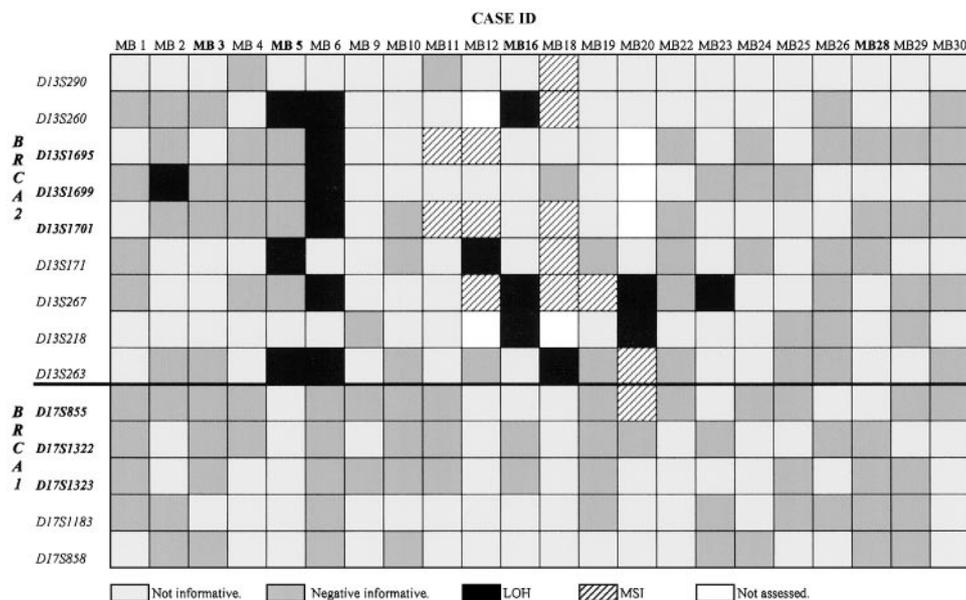
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CASE ID

Fig. 4. Patterns of LOH at *BRCA1* and *BRCA2* in 22 MBC cases. *Rows*, genetic markers; *lanes*, the cases analyzed. *Labels in bold*: intragenic loci, cases with germ-line mutations (*BRCA1*: *MB5*; *BRCA2*: *MB3*, *MB16*) and with *BRCA2* allele expression imbalance (*MB28*).

of 22 tumors (Fig. 3). However, further testing at *BAT26* (data not shown) did not reveal MSI at this sensitive mononucleotide repeat (38). This suggests that the MSI observed in the present MBC series might reflect spontaneous mutations at dinucleotide repeats, rather than tumor-associated mismatch repair deficiency (49–51).

In female patients, *BRCA1*-related tumors are characterized by poor differentiation and low ER/PR and c-erbB-2 positivity, suggesting independence from hormone receptors or c-erbB-2-mediated stimulation (23, 25–31). In contrast, a heterogeneous phenotype with high rates of positivity for ER/PR and for c-erbB-2, undistinguishable from that of *BRCA1/BRCA2*-unrelated tumors, has been reported in FBCs arising in *BRCA2* mutation carriers (28–31). At present, little is known of the immunophenotypic characteristics of MBCs stratified according to *BRCA1/BRCA2* mutation status. We obtained data on ER/PR, c-erbB-2, and Ki-67/MIB1 immunostaining for 19 cases of the present series, including all of the ascertained mutation carriers (Table 1). Thirteen tumors (68%) were ER/PR+, 1 (5%) was positive only for ER, 3 tumors (16%) were c-erbB-2+, 4 (21%) were Ki-67/MIB1+, one (5%) was negative for all of the markers tested. Interestingly, the cases with germ-line mutations included all of the c-erbB-2+ tumors in the series ($P = 0.004$), which corresponded to the *BRCA2* mutation carriers. Furthermore, two of these tumors were ER/PR− and Ki-67/MIB1+. The case with imbalance in *BRCA2* allele transcript expression (MB28) and the case with somatic loss of *BRCA2* (MB6) were ER/PR+, c-erbB-2−, and Ki-67/MIB1−. The association of germ-line *BRCA2* coding sequence mutations with c-erbB-2 positivity is consistent with the occurrence of one of the three *BRCA2* mutations in a case of Paget's disease (Fig. 2). In fact, a strong association between c-erbB-2 positivity and mammary and extramammary Paget's disease has been reported in the literature, suggesting that the c-erbB-2 protein might be implicated in promoting the *in vivo* intraepithelial spread of adenocarcinoma cells (44–45).

In conclusion, the prevalence of *BRCA1* and *BRCA2* mutations that we have found in this MBC series from Central Italy is relatively high. Mutations were associated with FH of breast/ovarian cancer. In addition, a marked germ-line *BRCA2* transcript imbalance and a somatic LOH at *BRCA2*, detected among mutation-negative cases with a low *a priori* mutation probability, suggest an even more relevant role of *BRCA2* as a cause of MBC at the population level. BCs arising in germ-line *BRCA2* mutation carriers tended to manifest a distinct

phenotype, characterized by high histological grade and c-erbB-2 positivity. This phenotype might imply a unique mechanism of molecular pathogenesis and is indicative of aggressive behavior. Overall, our data suggest that specific phenotypic characteristics could significantly contribute to the identification of *BRCA2*-related MBCs and that these tumors might benefit from *ad hoc* therapeutic approaches.

ACKNOWLEDGMENTS

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