Immunohistochemical classification of non-*BRCA1/2* tumors identifies different groups that demonstrate the heterogeneity of *BRCAX* families

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Around 25% of hereditary breast and ovarian cancer families have mutations in the *BRCA1* and *BRCA2* genes. The search for other genes has until now failed, probably because there is not one single *BRCAX* gene, but rather various genes that may each be responsible for a small number of breast cancer families and/or may interact according to a polygenic model. We have studied 50 tumors from probands belonging to non-*BRCA1/2* breast cancer families (*BRCAX*), using 25 immunohistochemical markers. The objective was to classify these tumors and confirm that they are heterogeneous. Unsupervised cluster analysis showed the existence of the following two main groups of tumors: high-grade and estrogen receptor (ER)-negative tumors (50%), and low-grade and ER-positive tumors (50%). In addition we identified five subgroups, three among the high-grade and two among the low-grade groups; one overexpressing HER-2 (18%); one with a basal-like phenotype (14%); one with a normal breast-like phenotype (18%); a luminal A subgroup (36%), and a luminal B subgroup (14%). Hypermethylation of the *BRCA1* gene was observed in 42% of the cases, spread across all five subgroups, but only 37% of those had loss of heterozygosity as well. These latter cases were all clustered in the high-grade group and the majority of them in the basal-like subgroup. Our results show that familial non-*BRCA1/2* tumors are heterogeneous and suggest a polygenic model for explaining the majority of *BRCAX* families. In addition we have defined a subset of them that have somatic inactivation of the *BRCA1* gene.
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More than a decade has passed since *BRCA1* and *BRCA2* were cloned[1,2] and their association with familial breast and ovarian cancer (FBOC) was established.[3] However, recent data indicate that these two genes explain only 25% of these families.[4–6]

The large number of families without an identified causative gene mutation has led many a groups to pursue putative *BRCAX* gene(s) through different approaches, but without success. Several reports have been published since 1995 suggesting linkage of the *BRCAX* gene to specific chromosomal regions,[7,8] but these data have not been reproduced in larger series.[9–11] Hedenfalk *et al*[12] carried out a study using expression arrays in a small group of non-*BRCA1/2* tumors, and concluded that they were heterogeneous and could be split into two main groups; but again, these results have not been reproduced. It has also been suggested that *BRCAX* families could be explained by a polygenic model,

or that they might carry mutations in another gene or genes conferring a moderately increased risk of breast cancer.[13–15]

Using paraffin-embedded tissue, we and others have previously demonstrated that *BRCA1* and *BRCA2* tumors can be differentiated because they have a specific immunohistochemical profile.[16–20] Based on these results, we hypothesized that the use of different immunohistochemical markers might help to group the non-*BRCA1/2* tumors, and to confirm that they are heterogeneous. The confirmation of this heterogeneity and their classification would be very important for further studies searching for candidate genes.

Materials and methods

Patients

We collected paraffin-embedded tumor tissues from 50 individuals (mean age 47 years) from 50 different high-risk families, who were studied for mutations in the *BRCA1* and *BRCA2* genes. These individuals were from families with at least three members affected with breast and/or ovarian cancer, at least one of whom was younger than 50 when diagnosed.[4] All 50 individuals were screened for mutations, including large deletions, in the *BRCA1* and *BRCA2* genes, and no mutations were detected.[4,21] The complete coding sequence and exon–intron boundaries of the BRCA1 and BRCA2 genes were analyzed by a combination of the following different techniques, depending on the center of origin: SSCP, PTT, CSGE, DGGE, and direct sequencing.[21]

We compared the profiles of non-*BRCA1/2* tumors *vs* a group of 33 tumors from patients carrying a mutation in the *BRCA1* gene, selected using the same criteria and studied with the same methodology as described above. Finally, we included a control group of 50 sporadic tumors that were selected because they were diagnosed at similar ages to the non-*BRCA1/2* tumors (mean 49 years). In order to confirm that the non-*BRCA1/2* and sporadic cases were genetically different, we estimated *BRCA1* and *BRCA2* carrier probabilities for both the groups using BRCAPRO.[22] Data on the majority of the markers assessed in the present study have been previously reported for all three groups of tumors.[18]

Tissue Microarray Construction

The morphological subtype and grade was assessed in complete sections of each tumor stained with hematoxylin–eosin (H–E). The non-*BRCA1/2* tumors consisted of 44 invasive ductal carcinomas, five *in situ* ductal carcinomas and one invasive lobular carcinoma.

Representative areas of the different lesions were carefully selected on H–E sections and marked on individual paraffin blocks. Two tissue cores (1 mm

in diameter) were obtained from each specimen. In addition, four cores of normal breast tissue and two cores of tonsil were included as controls. The tissue cores were arrayed onto one independent new paraffin block using a tissue microarray (TMA) workstation (Beecher Instruments, Silver Spring, MD, USA). The final TMA consisted of 106 cores, each 1 mm in diameter, spaced 0.8 mm from each other. A section stained with H–E was studied to confirm the presence of morphologically representative areas of the original lesions. The *BRCA1* and sporadic tumors were included in two separate TMAs using the same technique.

Immunohistochemical Studies

Immunohistochemical staining was performed by the Envision method (Dako, Glostrup, Denmark), with a heat-induced antigen retrieval step. Sections from the tissue array were immersed in 10 mM boiling sodium citrate at pH 6.5 for 2 min in a pressure cooker. Antibodies, dilutions and suppliers are listed in Table 1.

Between 150 and 200 cells per core were scored for the percentage of positive nuclei or cytoplasms, depending on the marker. We evaluated nuclear staining for estrogen receptor (ER); progesterone receptor (PR); p53; Ki-67; cyclins D1, D3, E, and A; p16; p27; p21; Skp2; retinoblastoma protein (Rb); E2F6; MDM2; topoisomerase IIα; survivin; and CHEK2, and evaluated cytoplasmic staining for BCL2, vimentin, cytokeratin 5/6 (CK5/6), cytokeratin 8 (CK8), and cyclin B1, as previously described.[16–18] Only the percentage of stained cells was considered (independent of the intensity), and the positivity threshold for each marker is listed in Table 1. We and others have previously used the same threshold in the analysis of these markers.[16,18,20,23–27] HER-2 expression was evaluated according to the four-category (0–3 +) DAKO system proposed for the evaluation of the HercepTest, and HER-2 expression of 3 + was the only value considered positive, as previously described.[18,19]

Statistical Analysis

Hierarchical unsupervised cluster analysis was performed by means of the UPGMA (unweighted pair-group method using arithmetic averages) method using correlation distance and Euclidean distance between markers. The cluster was displayed using SOTAARRAY[28] (software available at http://gepas.bioinfo.cipf.es/). The method was implemented in the GEPAS package.[29] Immunohistochemical results were represented by a range of color from green to red, the most green representing the lowest, and the most red the highest percentage of positive cells for each marker. Exceptions were grades which were scaled as 33% 'expressed' for grade 1, 66% for grade 2, and 100% for grade 3 and HER-2, which

Table 1 Antibodies used in the present immunohistochemical study and threshold to consider a tumor as positive, used in χ^2 analysis

Antibody	Clone	Dilution	Supplier	Threshold (%)
ER	1D5	1:30	Novocastra	10
PR	1A6	1:30	Novocastra	10
BCL2	124	1:80	DAKO	70
Ki-67	MIB1	1:30	DAKO	0–5/6–25/>25
p53	DO-7	1:50	Novocastra	25
HER-2	Herceptest	Prediluted	DAKO	3+
Cyclin D1	DCS-6	1:100	DAKO	30
Cyclin D3	DCS-22	1:10	Novocastra	[a]
Cyclin E	13A3	1:10	Novocastra	[a]
Cyclin A	6E6	1:100	Novocastra	[a]
Cyclin B1	7A9	1:25	Novocastra	[a]
p21	EA10	1:50	Oncogene	[a]
p16	Poly mouse	1:50	Santa Cruz	50
p27	57	1:1000	Transduction Lab	50
Skp2	1G12E9	1:10	ZYMED	[a]
Rb	G3–245	1:250	BD PharMingen	[a]
E2F6	Poly goat	1:50	Santa Cruz	[a]
CHEK2	DCS-270	1:25	Novocastra	60
Topoisomerase IIα	Ki-S1	1:400	DAKO	[a]
MDM2	IF2	1:10	Oncogene	[a]
CK 5/6	D5/16 B4	1:25	DAKO	[a]
CK 8	35BH11	1:10	DAKO	80
Vimentin	V9D	1:500	DAKO	[a]
Survivin	Poly rabbit	1:1000	RD Systems	[a]
EGFR	EGFR.113	1:10	Novocastra	[a]

ER, estrogen receptor; PR, progesterone receptor; HER-2, human epidermal growth factor receptor-2; EGFR, epidermal growth factor receptor.
[a]Any positive cell.

was scaled as for 100% for positive $(3+)$, and 0% for negative (Figure 1). We used the CAAT software based on Silhouette Width for clustering validation (software available at http://gepas.bioinfo.cipf.es/).

The χ^2-test was performed to determine the differences in the distributions of the expression of each antibody and grade between the groups (Tables 2 and 3). The statistical program SPSS 13.0 for Windows (SPSS Inc., Chicago, IL, USA) was used for this analysis. Correction for multiple testing was made by a permutation method in which group membership was randomly assigned, conserving observed proportions, and the distribution of minimum *P*-values determined over 10 000 permutations. Differences in median BRCAPRO probabilities were tested using the Mann–Whitney rank-sum test.

BRCA1 Promoter Hypermethylation

DNA methylation patterns in the CpG islands of the promoter of the *BRCA1* gene were determined by methylation-specific PCR in primary tumors after bisulfite treatment of DNA.[30] Placental DNA treated *in vitro* with *Sss*I bacterial methylase was used as a positive control, and DNA from normal lymphocytes was used as a negative control for methylated alleles of *BRCA1*.

BRCA1 Loss of Heterozygosity

Loss of Heterozygosity (LOH) analysis of the *BRCA1* gene was performed using the intronic microsatel-

lite markers D17S1322 and D17S855 that localize to introns 19 and 20, respectively, and D17S1327 that localizes in 17q21.31 outside the *BRCA1* gene.[31] The forward primer for each set was labeled using the fluorescent dye FAM (Applied Biosystems/PE Biosystems, Foster City, CA, USA). Reactions were cycled as follows: 95°C for 5 min, then 35 cycles at 94°C for 60 s, 55°C for 60 s, and 72°C for 90 s, followed by final elongation at 72°C for 5 min.

Allele sizes were determined using an automated capillary sequencer (ABI Prism™ 310; Applied Biosystems, Perkin Elmer, Warrington, UK) and were analyzed using GeneScan 3.1 software (Applied Biosystems, Warrington, UK). LOH was determined when the difference between peaks representing alleles in the tumor and the corresponding normal DNA reactions exceeded 25%.

Results

The morphological and immunohistochemical profiles of non-*BRCA1/2* tumors were established by analyzing grade and 25 immunohistochemical markers in 50 such tumors, and comparing them with 50 sporadic tumors. Non-*BRCA1/2* tumors were of lower grade (adjusted $P = 0.04$); 54% were grade 1 *vs* 20% of sporadic tumors (Table 2). Although there was marginal evidence that p53 and p21 expression differed between the two groups, these associations disappeared after correction for multiple testing. Overall, the expression of markers related to proliferation, cell cycle, apoptosis, hormone recep-
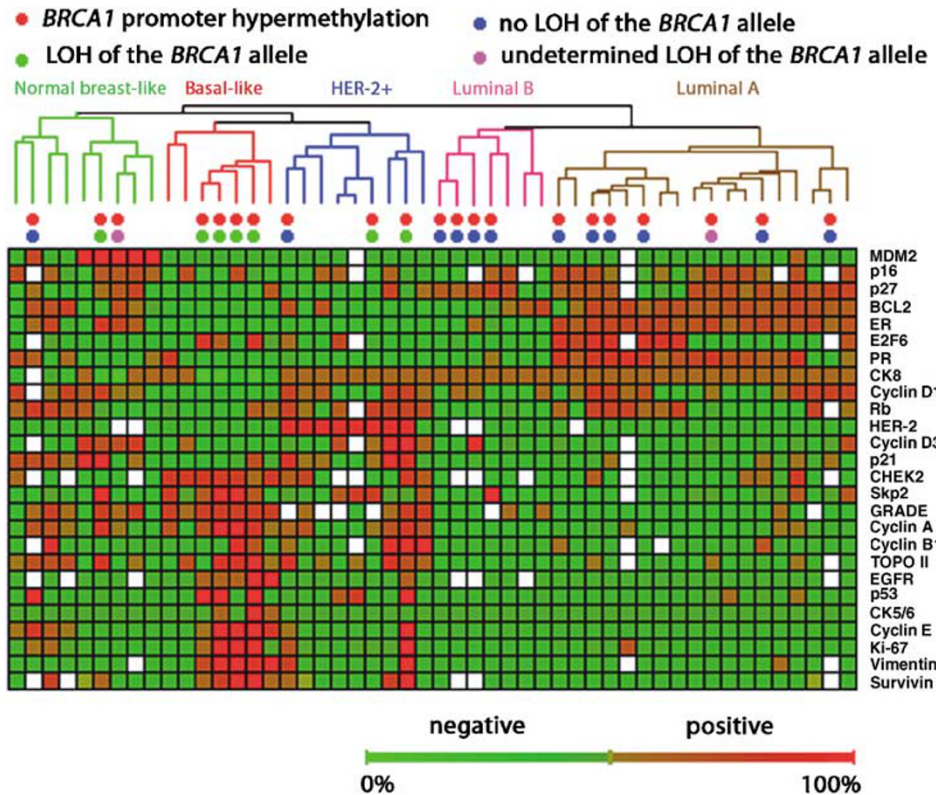
Figure 1 Hierarchical clustering of 50 non-BRCA1/2 tumors. White squares correspond to data not available. The percentage of positive cells for each immunohistochemical marker is represented as a range of color between the most green (lowest percentage) and the most red (highest percentage). Intermediate colors represent percentages between the lowest and the highest.

tors, and epithelial proteins showed similar patterns after adjustment for multiple testing (Table 2).

Although the phenotype of both groups was similar, they were genetically very different. Using BRCAPRO we found that the median probability of not being carrier of *BRCA1* or *BRCA2* mutations was 0.994 (range 0.964–0.997) for the sporadic tumors and 0.519 (range 0.062–0.987) for the non-*BRCA1/2* tumors ($P < 0.0001$).

Hierarchical Unsupervised Cluster Analysis

We performed a hierarchical unsupervised cluster analysis of the non-*BRCA1/2* tumors based on the 25 immunohistochemical markers and grade, and found that the 50 tumor samples were separated into two main groups with 25 tumors each, differentiated primarily by grade and ER status. The high-grade branch (Figure 1, left) included tumors of grade 2 or 3 (brown and red squares, respectively) that were ER negative and had overexpression of proteins that promote cell cycle progression and proliferation. The low-grade branch (Figure 1, right) included grade 1 tumors (green squares) that were ER positive and showed overexpression of proteins related to the inhibition of the cyclin–CDK complexes, or the overexpression of luminal epithelial proteins such as CK8.

Within the high-grade branch we distinguished the following three different subgroups: one characterized by HER-2 overexpression (Figure 1, blue branch) that corresponded to 18% of all cases; a second basal-like subgroup containing 14% of the cases; and a third group that represents 18% of all cases, defined by low expression of the luminal epithelial marker CK8 and overexpression of other proteins associated with cell cycle progression and proliferation. Furthermore, this latter subgroup contained most of the ER-positive tumors in this predominantly ER-negative branch (Figure 1, green branch), and we have named it the 'normal breast-like' group because of its similarity to the group described by Sorlie *et al*[32] with this name.

The low-grade branch could be divided into two further subgroups (Figure 1, pink and brown branches). The brown branch in Figure 1 was the largest (18 tumors) and demonstrated higher expression of ER, BCL2, CK8, and proteins that inhibit cell cycle progression. The second subgroup contained the remaining seven (14%) cases (Figure 1, pink branch) and showed low, or loss of, expression of ER, PR, and BCL2, but conserved the expression of CK8. We have named them the luminal A and B groups, respectively, according to Sorlie's classification.[32]

We performed an unsupervised cluster analysis with the 50 sporadic breast tumors, using the same

Table 2 Comparison between non-*BRCA1/2* tumors and a group of sporadic breast carcinomas of the number (and percentage in parenthesis) of positive cases for each immunohistochemical marker, unless otherwise indicated

	Non-*BRCA1/2* (n = 50) (%)	P	Sporadic (n = 50) (%)
Grade			
1	24 (54.5)		10 (20.0)
2	9 (20.5)		17 (34.0)
3	11 (25.0)	**0.002**[a]	23 (46.0)
Ki-67			
0–5%	35 (70.0)		26 (53.1)
6–25%	9 (18.0)		18 (36.7)
>25%	6 (12.0)	0.1	5 (10.2)
ER	30 (60.0)	0.3	33 (68.8)
PR	28 (56.0)	0.8	27 (55.1)
BCL2	23 (46.0)	0.8	22 (45.8)
p53	7 (14.0)	**0.041**[a]	15 (31.3)
HER-2 (3+)	8 (17.8)	0.9	9 (18.4)
Cyclin D1	28 (56.0)	0.9	28 (56.8)
Cyclin D3	18 (38.3)	0.1	26 (52.0)
Cyclin E	11 (22.0)	0.6	9 (18.0)
Cyclin A	20 (40.0)	0.4	23 (46.9)
Cyclin B1	9 (19.6)	0.3	6 (12.2)
p16	29 (67.4)	0.8	34 (69.4)
p21	18 (36.7)	**0.042**[a]	9 (18.4)
p27	27 (55.1)	0.1	34 (69.4)
Skp2	23 (46.9)	0.7	22 (44.0)
Rb	33 (68.8)	0.1	41 (82.0)
E2F6	17 (34.0)	0.8	17 (34.0)
CHEK2	9 (22.5)	0.6	12 (27.3)
Topo IIα	20 (41.7)	0.8	22 (44.0)
MDM2	7 (14.6)	0.8	8 (16.0)
CK5/6	3 (6.0)	0.1	7 (14.9)
CK8	39 (79.6)	0.8	38 (77.6)
Vimentin	8 (17.0)	0.3	12 (25.5)
Survivin	13 (28.9)	0.5	16 (34.8)
EGFR	6 (14.0)	0.8	7 (14.9)

ER, estrogen receptor; PR, progesterone receptor; HER-2, human epidermal growth factor receptor-2; EGFR, epidermal growth factor receptor.
Unadjusted *P*-value for the comparison between non-*BRCA1/2* and sporadic tumors.
[a]After corrections for multiple comparisons only the association with grade remained significant ($P < 0.04$).

immunohistochemical markers, and identified the same five subgroups, with the same pattern of immunohistochemical expression, and similar proportions in each subgroup; 16% HER-2 positive; 15% basal like; 11% normal breast like; 42% luminal A; and 16% luminal B. We validated these results using the Silhouette Width technique (data not shown).

Somatic Inactivation of the *BRCA1* Gene

We observed promoter hypermethylation of *BRCA1* in 21 (42%) of the 50 cases, and these were distributed evenly over the two main groups (Figure 1). There was no evidence of an association between hypermethylation and grade, or any of the immunohistochemical markers studied.

We tested for LOH at the *BRCA1* locus in 19 of the 21 cases with promoter hypermethylation of *BRCA1*

(there was no DNA from lymphocytes available for the other 2 cases with promoter hypermethylation), and observed LOH in seven (37%). All seven tumors with double somatic *BRCA1* inactivation were in the high-grade branch and four (57%) of those were in the basal-like group (Figure 1).

Discussion

Immunohistochemical analysis of *BRCA1* and *BRCA2* tumors has previously shown a good correlation between genotype and phenotype,[16,19,20] but there are very few studies on non-*BRCA1/2* tumors.[18,20,27,33] We have previously reported that non-*BRCA1/2* tumors tend to be grade 1–2 and ER and PR positive, have a low proliferative index, and express p53 to a similar extent to *BRCA2* and sporadic tumors, but less than *BRCA1* tumors.[18–20] The present study has confirmed our previous results with new markers related to cell cycle, apoptosis, proliferation, stromal, and epithelial markers. We can say that non-*BRCA1/2* tumors are of lower grade than sporadic tumors, but they are quite similar with respect to the immunohistochemical markers studied (Table 2).

Immunohistochemistry Classification of Familial Non-*BRCA1/2* Tumors

By using a hierarchical unsupervised cluster analysis with 25 markers and grade, we have established a classification of the non-*BRCA1/2* tumors that demonstrates their heterogeneity. Non-*BRCA1/2* tumors can be divided into two main groups primarily according to their ER status and grade. The first group is characterized by higher grade; ER negativity; and the expression of proteins related to proliferation and cell cycle progression, and the second group by low grade; ER positivity; and overexpression of some cyclin–CDK complex inhibitors, antiapoptotic, and luminal (CK8) proteins (Figure 1).

The two main groups of our analysis can be further divided into five subgroups that are consistent with the classification system established by Sorlie *et al*[32] in sporadic breast cancer, using a cDNA array study, as follows: (1) HER-2 positive; (2) basal like; (3) normal breast like, (4) luminal A, and (5) luminal B. In addition, we performed an unsupervised cluster analysis of 50 sporadic tumors using the same immunohistochemical markers used in the non-*BRCA1/2* group, and obtained similar subgroups. Thus, we conclude that non-*BRCA1/2* tumors are heterogeneous and that the immunohistochemical classification is very similar to that found for sporadic tumors using both expression arrays[32] and immunohistochemical markers. We obtained consistent results after excluding families with any ovarian or male breast cancers (data not shown).

Table 3 Immunohistochemical markers that significantly differentiate non-BRCA1/2 tumors with and without somatic BRCA1 inactivation (promoter hypermethylation and LOH of the BRCA1 allele), and comparisons with BRCA1 tumors

	Non-BRCA1/2 tumors without somatic BRCA1 inactivation (n = 41) (%)	P*	Non-BRCA1/2 tumors with somatic BRCA1 inactivation (n = 7) (%)	P**	BRCA1 tumors (n = 33) (%)	P***
Grade						
1	23 (63.9)		0	0.2	0	
2	8 (22.2)		0		5 (16.1)	
3	5 (13.9)	<0.001	6 (100.0)		26 (83.9)	<0.001
ER						
Negative	14 (34.1)		6 (85.7)	0.4	23 (71.9)	
Positive	27 (65.9)	0.011[a]	1 (14.3)		9 (28.1)	0.001
PR						
Negative	15 (36.6)		7 (100.0)	0.1	26 (78.8)	
Positive	26 (63.4)	0.002	0		7 (21.2)	<0.001
BCL2						
Negative	20 (48.8)		7 (100.0)	0.2	28 (84.8)	
Positive	21 (51.2)	0.012[a]	0		5 (15.2)	0.001
Ki-67						
0–5%	32 (78.0)		1 (14.3)	0.6	9 (27.3)	
6–25%	7 (17.1)		2 (28.6)		11 (33.3)	
>25%	2 (4.9)	<0.001	4 (57.1)		13 (39.4)	<0.001
p53						
Negative	38 (92.7)		3 (42.9)	0.6	17 (51.5)	
Positive	3 (7.3)	0.001	4 (57.1)		16 (48.5)	<0.001
Cyclin E						
Negative	35 (85.4)		2 (28.6)	0.3	15 (46.9)	
Positive	6 (14.6)	0.001	5 (71.4)		16 (53.1)	<0.001
Cyclin A						
Negative	29 (70.7)		0	0.3	4 (12.1)	
Positive	12 (29.3)	<0.001	7 (100.0)		29 (87.9)	<0.001
p27						
Negative	16 (40.0)		6 (85.7)	0.2	21 (63.6)	
Positive	24 (60.0)	0.025[a]	1 (14.3)		12 (36.4)	0.044[a]
Skp2						
Negative	25 (62.5)		0	0.2	5 (15.6)	
Positive	15 (37.5)	0.002	7 (100.0)		27 (84.4)	<0.001
CHEK2						
Negative	26 (83.9)		3 (42.9)	1.0	12 (42.9)	
Positive	5 (16.1)	0.021[a]	4 (57.1)		16 (57.1)	0.001
CK5/6						
Negative	40 (97.6)		5 (71.4)	0.4	19 (57.6)	
Positive	1 (2.4)	0.008[a]	2 (28.6)		14 (42.4)	<0.001
CK8						
Negative	4 (10.0)		5 (71.4)	0.4	18 (54.5)	
Positive	36 (90.0)	<0.001	2 (28.6)		15 (45.5)	<0.001
Vimentin						
Negative	35 (92.1)		2 (28.6)	0.3	15 (46.9)	
Positive	3 (7.9)	<0.001	5 (71.4)		17 (53.1)	<0.001
Survivin						
Negative	29 (80.6)		1 (14.3)	0.1	12 (42.9)	
Positive	7 (19.4)	<0.001	6 (85.7)		16 (57.1)	0.002
EGFR						
Negative	33 (97.1)		2 (28.6)	0.2	15 (53.6)	
Positive	1 (2.9)	<0.001	5 (71.4)		13 (46.4)	<0.001

ER, estrogen receptor; PR, progesterone receptor; EGFR, epidermal growth factor receptor.
[a]No longer significant after correcting for multiple testing.
*P-value for comparison between non-*BRCA1/2* without and with somatic *BRCA1* inactivation.
**P-value for comparison between non-*BRCA1/2* with somatic *BRCA1* inactivation and *BRCA1* tumors.
***P-value for comparison between non-*BRCA1/2* without somatic *BRCA1* inactivation and *BRCA1* tumors.

1304

Somatic Inactivation of the *BRCA1* Gene

We found that 42% of the non-*BRCA1/2* tumors presented promoter hypermethylation of the *BRCA1* gene, a percentage that is higher than in sporadic cases, where it ranges between 11 and 30%.[34,35] However these tumors were evenly distributed across both groups and hypermethylation was not associated with estrogen or progesterone receptor status, or (higher) grade, as has been suggested by some authors.[35,36] Seven (37%) of the non-*BRCA1/2* tumors with promoter hypermethylation also had LOH at, and therefore total inactivation of, *BRCA1.* All seven were all in the high-grade group and the majority showed the basal-like phenotype (Figure 1), the latter being characteristic of *BRCA1* tumors.[37–40] Although the sample size was small, we compared this subgroup with the rest of the non-*BRCA1/2* tumors and found significant differences for the majority of the markers based on unadjusted *P*-values (Table 3). Consistent differences were observed when 33 *BRCA1* tumors were compared with the latter group using previously published data.[18] However, the same comparison between the seven *BRCA1/2* tumors with somatic *BRCA1* inactivation and the 33 *BRCA1* tumors did not reveal significant differences even before correction for multiple testing (Table 3). In addition, six out of the seven tumors with *BRCA1* promoter hypermethylation and LOH showed morphologic characteristics of medullary carcinoma, a subtype associated with *BRCA1* tumors.[41] All these observations suggest that in the majority of cases, a double somatic 'hit' in the *BRCA1* gene is necessary for non-*BRCA1/2* tumors to generate a *BRCA1* phenotype; the first hit would be promoter hypermethylation and the second hit an LOH of the wild-type allele, and both would occur early in tumorigenesis in order to be able to mimic the immunohistochemical profile of *BRCA1* tumors.

Genetic Implications of the New Familial Non-*BRCA1/2* Tumor Classification

The classification by immunohistochemistry of non-*BRCA1/2* tumors into five subgroups confirms that non-*BRCA1/2* tumors constitute a heterogeneous group of tumors, and it supports the hypothesis that the majority of familial non-*BRCA1/2* tumors might be explained by a polygenic model (that is, multiple low-penetrance genes),[15] rather than by a single *BRCAX* gene. That is, our results could represent a practical validation of this hypothesis because we have found that non-*BRCA1/2* tumors have the same immunohistochemical profile as that described in sporadic breast tumors, a type of tumor classically associated with a polygenetic model. This concept does not exclude the existence of some genes that could each explain a small number of families,[13,14] as was recently shown for *CHEK2*.[42,43] In fact, our classification could be very useful in defining more homogenous groups for linkage and other studies designed to identify such genes.

Clinical Implications of the *BRCA1*-like Group

The same mechanisms of somatic inactivation of the *BRCA1* gene that we have described in 14% of our cases was recently observed in a group of sporadic breast tumors,[35] and these tumors were also high grade and ER negative. These data confirm the specific characteristics (high grade and ER negative) of this group of cases that exhibit what has generically been named 'BRCAness', and that both sporadic[44] and familial breast tumors can present as 'BRCA like'. Some experimental studies using demethylating agents are now being conducted on different tumors with good results,[45] and this opens up a new avenue for the treatment of tumors with an allele inactivated by hypermethylation that has to be explored further. On the other hand, the identification of this group of tumors with 'BRCAness' also provides a basis for new therapeutic strategies based on the sensitivity to DNA-damaging agents that *BRCA1* tumors present.[46] DNA repair protein PARP inhibitors and DNA crosslinking agents (cisplatin, mytomycin C, diepoxibutane) seem to affect tumor cells with *BRCA1* mutations by inhibiting the DNA repair of single strand breaks and increasing their non-viability, while leaving normal cells or cells with a functional *BRCA1* allele unaffected.[47] Therefore, *BRCA1* tumors occurring as a result of constitutional or somatic mutations could represent a new group for targeted therapeutic strategies.

Disclosure/conflict of interest

The authors declare that they have no conflict of interest.

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1306

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