

Phenotypic characterization of *BRCA1* and *BRCA2* tumors based in a tissue microarray study with 37 immunohistochemical markers

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Key words: basal phenotype, *BRCA1*, *BRCA2*, cell cycle, immunohistochemistry, tissue microarray

Summary

Familial breast cancers that are associated with *BRCA1* or *BRCA2* germline mutations differ in both their morphological and immunohistochemical characteristics.

To further characterize the molecular difference between genotypes, the authors evaluated the expression of 37 immunohistochemical markers in a tissue microarray (TMA) containing cores from 20 *BRCA1*, 14 *BRCA2*, and 59 sporadic age-matched breast carcinomas. Markers analyzed included, among others, common markers in breast cancer, such as hormone receptors, p53 and HER2, along with 15 molecules involved in cell cycle regulation, such as cyclins, cyclin dependent kinases (CDK) and CDK inhibitors (CDKI), apoptosis markers, such as BCL2 and active caspase 3, and two basal/myoepithelial markers (CK 5/6 and P-cadherin). In addition, we analyzed the amplification of *CCND1*, *CCNE*, *HER2* and *MYC* by FISH.

Unsupervised cluster data analysis of both hereditary and sporadic cases using the complete set of immunohistochemical markers demonstrated that most *BRCA1*-associated carcinomas grouped in a branch of ER-, HER2-negative tumors that expressed basal cell markers and/or p53 and had higher expression of activated caspase 3. The cell cycle proteins associated with these tumors were E2F6, cyclins A, B1 and E, SKP2 and Topo II α . In contrast, most *BRCA2*-associated carcinomas grouped in a branch composed by ER/PR/BCL2-positive tumors with a higher expression of the cell cycle proteins cyclin D1, cyclin D3, p27, p16, p21, CDK4, CDK2 and CDK1.

In conclusion, our study in hereditary breast cancer tumors analyzing 37 immunohistochemical markers, define the molecular differences between *BRCA1* and *BRCA2* tumors with respect to hormonal receptors, cell cycle, apoptosis and basal cell markers.

Introduction

It has recently been shown that cancers arising in *BRCA1* and *BRCA2* mutation carriers, differ in terms of their histopathologic features and that each of them differs from age-matched sporadic breast cancer as well [1–4]. Cancers associated with *BRCA1* are poorly differentiated infiltrating ductal carcinomas (IDCs), with higher mitotic counts and pleomorphism, and less tubule formation than sporadic tumors. In addition, more cases with the morphological features of typical or atypical medullary carcinoma are seen in these patients. When compared with sporadic age-matched controls,

breast carcinomas from *BRCA2* mutation carriers tend to be of a higher grade. The immunophenotypic features of breast carcinomas arising in *BRCA1* and *BRCA2* mutation carriers have been evaluated in some series [5–9]. Compared with age-matched controls, *BRCA1* tumors have been found to be more frequently estrogen receptor- (ER) and progesterone receptor-(PR) negative and p53-positive. Differences however are not usually found in *BRCA2*-associated tumors [7]. Furthermore, *BRCA1* and *BRCA2*-associated breast carcinomas show a low frequency of HER-2 expression and amplification [6, 8, 9].

Proliferation rate, gauged through mitotic counting and Ki67 immunostaining, was one of the most important characteristics differentiating familial and sporadic

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breast cancer [2, 10], suggesting that gene expression involved in cell cycle control differs between genotypes. Cell cycle progression is governed by cyclin dependent kinases (CDKs) that are activated by cyclin binding and inhibited by CDK inhibitors (CDKI) [11]. The passage from G₁ to S phase is regulated by the activities of cyclin D1/CDK4, cyclin E/CDK2, and cyclin A/CDK2 complexes. Cyclin B/CDK1 regulates the G₂-M transition. Two CDKI families regulate the cell cycle. Members of the CDK4 (INK4) inhibitor family such as p15^{INK4B} and p16^{INK4A}, inhibit and specifically bind to CDK4 and CDK6. In contrast, members of the kinase inhibitor protein (KIP) family (p21^{CIP1}, p27^{Kip1}, and p57^{Kip2}) have opposite effects on the function of different CDKs. Whilst p27 and p21 have a negative effect on E/CDK2 and cyclin A/CDK2 activity, they seem to activate cyclin D/CDK complexes [11].

The expression of these cell cycle proteins has been extensively studied in sporadic breast cancer and in the majority of cases each marker has been associated with specific morphological (histological grade) or biological (ER status) characteristics. However, hereditary breast cancer studies are uncommon and generally limited analyzing the expression of individual cell cycle proteins [5, 7, 12, 13].

Deregulation of apoptosis plays an important role in the pathogenesis and progression of breast cancer, as well as in the response of tumors to therapeutic intervention. Over-expression of BCL2 is commonly observed in ER-positive sporadic breast carcinomas and has been associated with good prognosis [14]. Compared with BCL2, far less is known about the expression of other apoptotic markers in breast tumors in general and in hereditary cases in particular.

Expression studies using conventional immunohistochemistry on whole sections [15–17], and also tissue microarray (TMA) immunohistochemistry [18–20] and cDNA array technology [21, 22] have demonstrated that there is a subset of breast carcinomas that express so-called “basal cell” or myoepithelial markers, such as high-molecular weight cytoqueratins (CK) CK 5/6, CK14 and CK17, and P-cadherin. These tumors are often both poorly differentiated and ER-negative, which are two commonly found features in *BRCAl*-associated breast carcinomas.

The aim of this study was to analyze cell cycle deregulation, apoptosis markers, and the basal cell phenotype in hereditary breast cancer. To this end, the expression pattern of several cyclins, CDKs and CDKIs, other cell cycle proteins, and basal cell markers was analyzed in a TMA containing a group of *BRCAl*, *BRCAl*, *BRCAl* and sporadic breast carcinomas. The results characterise the molecular differences between hereditary breast cancer genotypes yet further.

Patients and methods

Patients were obtained from three centers in Spain: Spanish National Cancer Center (CNIO) and the

Table 1. Antibodies used in the present immunohistochemical study

Antibody	Clone	Dilution	Supplier
ER	1D5	1:30	Novocastra
PR	1A6	1:30	Novocastra
BCL2	124	1:80	DAKO
Ki-67	MIB1	1:30	DAKO
P53	DO-7	1:50	Novocastra
HER-2 (IHC)	Herceptest		DAKO
E-Cadherin	4A2C7	1:200	Zymed
P-Cadherin	56	1:200	Transduction Labs
β -Catenin	14	1:1000	Transduction Labs
γ -catenin	15	1:1000	Transduction Labs
p120 ^{ctn}	98	1:500	Transduction Labs
MDM2	IF2	1:10	Oncogen
Topoisomerase II α	Ki-S1	1:400	DAKO
Cyclin D1	DCS-6	1:100	DAKO
Cyclin D3	DCS-22	1:10	Novocastra
CDK4	35.1	1:10	Chemicon
Cyclin E	13A3	1:10	Novocastra
Cyclin A	6E6	1:100	Novocastra
CDK2	8D4	1:500	NeoMarkers
Rb	G3-245	1:250	BD PharMingen
E2F6	Poly goat	1:50	Santa Cruz
Cyclin B1	7A9	1:25	Novocastra
CDK1	1	1:1500	Transduction Labs
p16	Poly mouse	1:50	Santa Cruz
p21	EA10	1:50	Oncogene
p27	57	1:1000	Transduction Lab
SKP2	1G12E9	1:10	ZYMED
BAX	Poly rabbit	1:750	Santa Cruz
BCLXL	2H12	1:10	Zymed
Survivin	Poly rabbit	1:1000	RD Systems
NFKB p65	F6	1:350	Santa Cruz
Active caspase 3	C92-605	1:25	BD PharMingen
CK 5/6	D5/16 B4	1:25	DAKO
CK 8	35BH11	1:10	DAKO
CAM 5.2	CAM 5.2	1:25	Becton Dickinson
Vimentin	V9D	1:500	DAKO
Chk 2	Poly rabbit	1:500	Supplied by E Salido

Fundación Jimenez Díaz in Madrid and the Hospital Sant Pau in Barcelona. They were selected from high risk breast cancer families with at least three women affected with breast and/or ovarian cancer, one of them younger than 50 years of age, or a male with breast cancer. The index case of each family was screened for

mutations in the BRCA1 and BRCA2 genes by a combination of SSCP, CSGE and PTT techniques. Some of these results have been previously reported [23].

The TMA characteristics were recently published [24]. It contains 34 IDC from 20 index patients with mutation in BRCA1 (mean age 42 years) and 14 in BRCA2 (mean age 42.58 years). The morphological characteristics of this series, evaluating whole histological sections, and some immunohistochemical features using 11 markers related with hormonal, proliferation and adhesion molecules markers are included in this study.

In addition to familial cases, 57 new infiltrating breast carcinomas from women without a family history of breast cancer (mean age 42.56) were studied, to give an age distribution similar to that of familial case subjects. In these cases, morphology was evaluated on whole histological sections and cores were obtained from selected areas to perform a control TMA for the immunohistochemical study.

Immunohistochemistry and fluorescence in situ Hybridization (FISH)

Immunohistochemical staining was performed by the LSAB method (Dako, Glostrup, Denmark) with a heat-induced antigen retrieval step. BAX, survivin, NFKB p65, CK 5/6, CK8, CAM 5.2 and vimentin were performed by the Envision method (DAKO). Sections from the tissue array were immersed in boiling 10 mM sodium citrate at pH 6.5 for 2 min in a pressure cooker. Antibodies, dilutions and suppliers are listed in Table 1.

Two pathologists (EH and JP) simultaneously evaluated the immunohistochemical staining in order to avoid observer subjectivity as much as possible. The percentage of stained nuclei was evaluated, independent of the intensity, for ER, PR, p53, Ki67, MDM2, topoisomerase II- α , cyclins D1, D3, E and A, CDK1, CDK2, CDK4, Rb, E2F6, p16, p21, p27, and SKP2. In the same way, the percentage of cells with cytoplasmic stain was scored for BCL2, CK5/6 and cyclin B1, CK8, CAM 5.2, vimentin and active caspase 3. Finally, BAX, BCLX, survivin, nuclear factor kappa B (NFKB/p65) were assessed taking in to account the staining intensity between 0 and 3 since 100% of cellular cytoplasm were stained in positive cases and we consider that it is the only way to distinguish between the different cases. Adhesion molecules, and HER2 were assessed as previously described [24].

FISH analysis was carried out for the detection of *CCND1* and *CCNE* gene amplification as previously reported for *HER2* and *c-MYC* [24]. For *CCND1* amplification we used the commercial probe from Vysis (Downer's Grove, IL), which spans the entire gene, and is labeled in SpectrumOrange. This probe also contains a centromeric probe for chromosome 11, which is labeled in SpectrumGreen and hybridizes to the alpha satellite DNA located at the centromere of chromosome 11 (11p11.1-q11.1). For *CCNE* amplification we used the Bacterial Artificial Chromosome (BAC) CTD-

2057O4, from Research Genetics, Invitrogen Corp., which spans the entire genomic region, together with a BAC RP11-198M12, from the Human BAC Clone Library RPC11 (Children's Hospital Oakland Research Institute, CA, USA) from the short arm chromosome 19 that was used as control of the ploidy level for chromosome 19.

FISH evaluation was performed by two investigators (SR and JCC) with no previous knowledge of the genetic, clinical or IHC results. Scoring of fluorescence signals was carried out in each sample by counting the number of single copy gene and centromeric signals in an average of 130 (60–210) well-defined nuclei. Amplification was defined as the presence (in >5% of tumor cells) of either >10 gene signals or more than three times as many gene signals as centromere signals of the chromosome. The cut-off values for the copy number changes were obtained from the analysis of normal adjacent cells in each experiment.

Statistical analysis

To compare the different immunohistochemical characteristics among the three groups of familiar breast cancer, two-sided Student's *t*-test was performed for continuum variables, and the Chi-square test for categorical variables. Non-adjusted *p*-values accounting for multiple testing were calculated using the step-down minP method of Westfall and Young [25]. Hierarchical unsupervised clustering was performed using the UP-GMA method. The statistical test and the clustering are implemented in the GEPAS package [26]. For clustering we use the percentage data as continuum. In case of grade, BAX, BCLX, Survivin, NFKBp65 we introduce 0, 1, 2, 3 as 0%, 33%, 66% and 100%. Adhesion molecules and HER-2 values were introduced for clustering as 100% when they were conserved or positive for HER-2 and 0% when they were reduced or negative, respectively.

Results

Immunohistochemistry

The immunophenotypes of BRCA1, BRCA2 and sporadic tumors are shown and contrasted in Table 2. Although 11 markers had been previously analysed as categorical variables [24], we have now included them as continuum variables for statistical comparisons between genotypes (Table 2) and cluster analysis (Figure 2). Representative immunohistochemical staining of several markers is included in Figure 1.

When BRCA1 carcinomas and BRCA2 carcinomas were compared, 15 out of 37 markers showed notable differences in their level of expression ($p < 0.05$) (Table 2). Markers more expressed in BRCA1 than in BRCA2 included p53, active caspase 3, vimentin and the basal cytokeratin CK5/6. In contrast to BRCA1,

Table 2. Comparison of immunohistochemical continuum variables among familial IDCs with BRCA1 and BRCA2 mutation and sporadic cases

	BRCA1 (n = 20) Mean ± SD	*p	BRCA2 (n = 14) Mean ± SD	*p	Sporadic vs. BRCA1 (n = 57) Mean ± SD	*p
Hormone receptors						
ER	12.75 ± 23.25	<0.001	58.57 ± 27.69	NS	44.82 ± 33.47	<0.001
PR	4.60 ± 7.54	0.006	25.71 ± 26.22	NS	35.53 ± 35.35	<0.001
Proliferation						
Ki-67	20.05 ± 17.91	NS	12.21 ± 11.43	NS	13.95 ± 14.56	NS
Topo II α	13.82 ± 19.16	NS	19.17 ± 17.16	NS(0.083)	9.32 ± 9.42	NS
p53	32.55 ± 35.17	0.006	6.50 ± 12.53	NS	13.95 ± 25.27	0.042
MDM2	0.53 ± 1.57	0.025	6.07 ± 10.59	NS	8.98 ± 19.48	0.003
Cell cycle						
Cyclin D1	4.20 ± 11.23	<0.001	43.57 ± 31.95	NS	35.58 ± 26.55	<0.001
Cyclin D3	2.9 ± 7.08	0.001	29.46 ± 28.46	NS(0.068)	12.11 ± 12.17	<0.001
CDK4	6.50 ± 18.43	0.001	46.92 ± 37.72	NS	28.39 ± 39.53	0.008
p16	40.00 ± 37.58	<0.001	86.92 ± 17.97	0.001	56.73 ± 35.64	NS
Cyclin E	15.79 ± 24.79	NS	7.50 ± 12.51	NS	8.51 ± 16.95	0.007
CDK2	4.37 ± 7.35	NS	10.64 ± 15.29	NS	3.86 ± 8.18	NS
p21	4.75 ± 5.49	NS(0.056)	8.46 ± 5.15	NS	10.88 ± 16.28	0.030
p27	37.50 ± 26.33	<0.001	72.14 ± 21.90	0.001	46.88 ± 25.29	0.001
SKP2	20.79 ± 13.77	NS	17.50 ± 19.68	NS(0.078)	6.75 ± 6.23	<0.001
Rb	12.94 ± 22.01	NS	14.58 ± 18.02	<0.001	41.49 ± 26.27	<0.001
E2F-6	25.00 ± 29.88	NS	11.92 ± 25.05	NS	4.82 ± 17.05	0.034
Cyclin A	19.75 ± 10.57	NS	14.67 ± 14.41	NS	8.16 ± 6.09	<0.001
Cyclin B1	11.00 ± 19.70	NS	5.38 ± 9.67	NS	3.13 ± 5.09	NS
CDK1	11.58 ± 24.04	NS	13.33 ± 22.59	NS	6.84 ± 18.81	NS
CHK2	40.63 ± 32.95	NS	40.00 ± 35.16	0.030	11.23 ± 23.60	0.005
Epithelial markers						
CK5/6	11.50 ± 23.90	0.014	0.71 ± 2.67	NS	2.72 ± 12.75	NS
CK8	63.50 ± 33.91	0.005	93.57 ± 24.05	NS	92.11 ± 20.50	0.005
Vimentin	36.50 ± 38.42	0.001	2.86 ± 10.69	NS	6.32 ± 19.76	0.010
CAM 5.2	9.41 ± 22.49	NS	24.17 ± 31.75	NS	27.02 ± 29.81	0.019
Apoptosis markers						
BCL2	18.75 ± 29.59	0.002	56.43 ± 33.13	NS(0.091)	38.68 ± 35.88	0.019
Active caspase 3	32.35 ± 34.73	<0.001	0.00 ± 0.00	NS	2.86 ± 12.75	0.008

*p (student *t*-test , statistical signification). NS (not statistically significant).

BRCA2 tumors expressed more extensively ER, PR, MDM2, different cell cycle markers, such as type D cyclins (D1 and D3), CDK4, and CDKIs (p16, p21 and p27), the apoptotic marker BCL2 and the luminal marker CK8. Differences between hereditary and sporadic cases are shown in Table 2.

Since the amount of information generated in this study using TMA was extensive and also the data multidimensional we used an unsupervised hierarchical clustering for data analysis. By applying this algorithm, the immunohistochemical markers were clustered according to the relevance of their expression pattern and the tumors based upon the similarity of expression of the different biomarkers. Hereditary tumors were divided into two main clusters (Figure 2a): one included all but two BRCA2 tumors and showed a broadly sim-

ilar ER-positive phenotype, including higher expression of PR, BCL2 and the cell cycle proteins cyclin D1, cyclin D3, p27, p16, p21, CDK4, CDK2 and CDK1. The second branch grouped most ER-negative carcinomas including all but three BRCA1 tumors. Tumors in this branch frequently expressed basal cell markers (P-cadherin and/or CK5/6) and/or p53. This group showed increased expression of the cell cycle molecules E2F6, cyclins A, B1 and E, SKP2 and Topo II α .

When all sporadic and hereditary cases were collectively analyzed (Figure 2B), two main branches clustered ER-positive and ER-negative tumors respectively, with a similar marker distribution and expression as observed in familial cases only. Whereas in the second cluster BRCA2 carcinomas were intermixed with sporadic tumors, most BRCA1

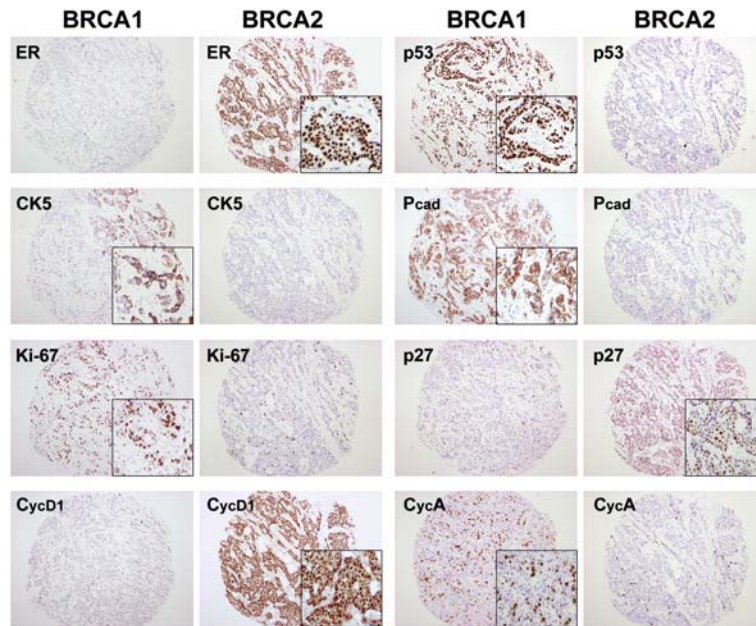


Figure 1. Immunohistochemical differences between BRCA1 and BRCA2 tumors assessed by tissue microarrays. Representative examples of two different tumors are shown. Note the distinct expression patterns of the markers used between BRCA1 (first and third columns) and BRCA2 tumors (second and fourth columns). ER, Estrogen receptor; CK5, Cytokeratin 5; Pcad, P-cadherin; CycD1, Cyclin D1; CycA, Cyclin A. Insets are magnification of selected core areas to show the staining pattern of each marker.

carcinomas in the first cluster were grouped in a main sub-branch including carcinomas that expressed basal cell markers and/or p53. This group of tumors was clearly separated from ER-negative breast carcinomas that overexpressed HER2, which were sporadic carcinomas.

Fish analysis

FISH study demonstrated a high percentage of cyclin D1 and MYC amplification in *BRCA2* carcinomas; however, the number of cases available was too low to be able to draw conclusions. We did not observe cyclin E amplification in any of the hereditary or sporadic cases studied. HER2 amplification was only observed in 22% of sporadic cases but in none hereditary tumor (Table 3).

Discussion

To characterize the phenotypic characteristics of *BRCA1* and *BRCA2* breast carcinomas in more detail, the expression of 37 immunohistochemical markers and the amplification status of 4 genes were investigated in primary IDCs from *BRCA1* and *BRCA2* mutation carriers and from age-matched control patients. This present study demonstrates the major differences in the immunohistochemical profile of different genotypes, not only in those markers previously analyzed in hereditary breast cancer, such as ER, PR, HER2 and p53, but also in those less commonly or perhaps never studied, including cell cycle, apoptosis, and basal cell markers. As previously

reported in other series, *BRCA1* tumors were characterized by their hormone receptor negativity and by a high frequency of p53 positivity, whereas a contrary phenotype was observed in *BRCA2* carcinomas [5–8]. Interestingly, 25% of *BRCA2* tumors showed MDM2 expression, a change that was not observed in *BRCA1* carcinomas. Although this finding could suggest deregulation of the p53 pathway by alternative mechanisms in the two genotypes, the associated expression of p21 tends to suggest the functional preservation of this pathway in *BRCA2* carcinomas.

Deregulation of cell cycle machinery is a common finding in breast cancer, and is frequently secondary to alterations in the proteins controlling the G1/S transition, including cyclins, CDKs and CDKIs [27]. In spite of their importance in tumor development, there is limited data available surrounding the expression of these cell cycle molecules in *BRCA1*- and *2*-associated breast cancer. For example, the expression of cyclin D1 has been reported in some series; however, for most of the other cell cycle markers, there is little or no previous information available [5, 7, 13]. The most striking differences between *BRCA1* and *BRCA2* hereditary breast carcinomas in the expression of cell cycles molecules were observed in type D (D1 and D3) cyclins, their associated CDK (CDK4), and CDKIs (p16, p21, p27), which were downregulated in *BRCA1* with respect to *BRCA2* carcinomas.

In support of our results, a lower incidence of cyclin D1 expression in *BRCA1* [7, 13, 28] than in *BRCA2* carcinomas or age-matched controls was observed in previous reports, using both- immunohistochemistry and cDNA microarrays. This is not surprising

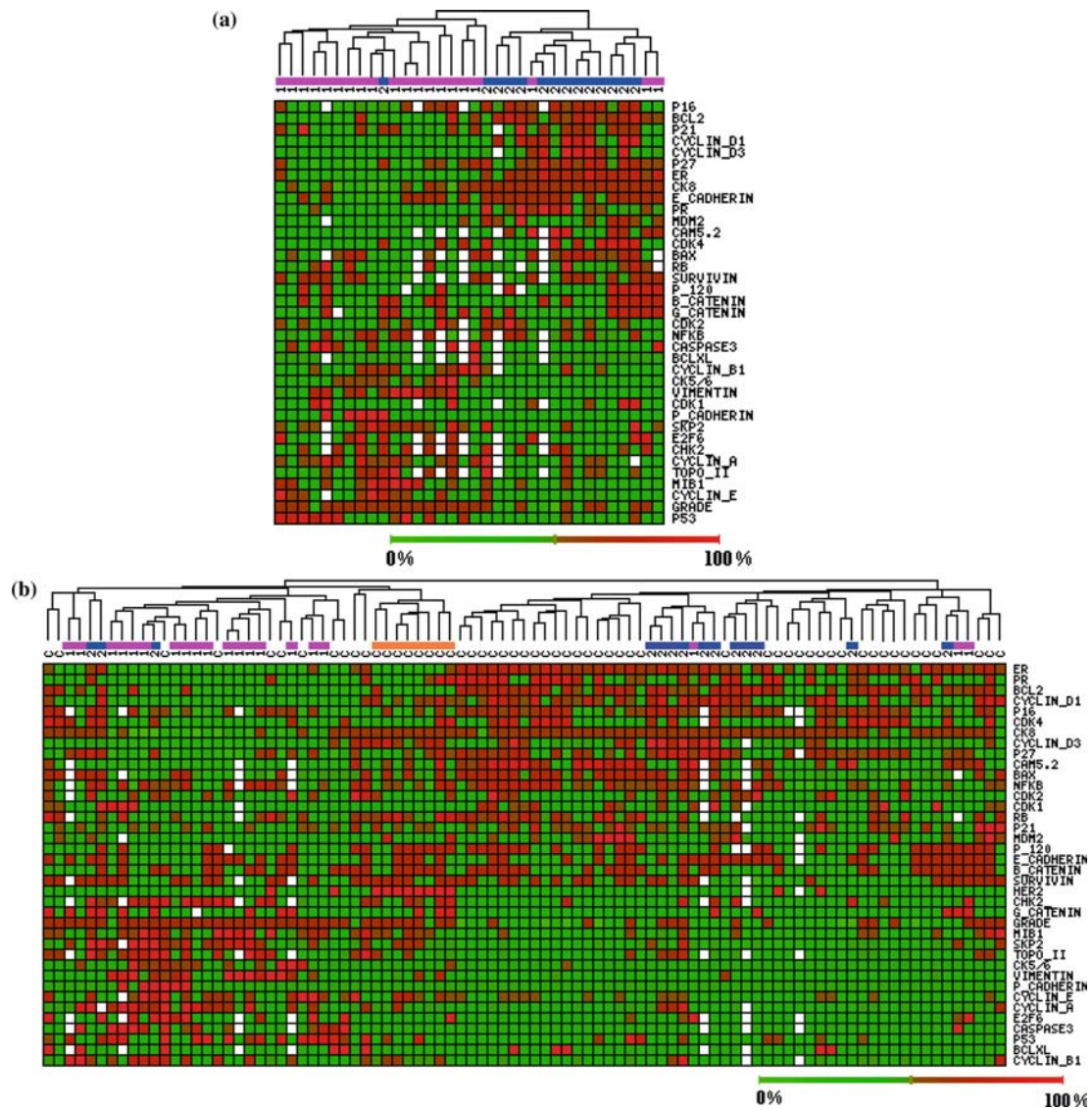


Figure 2. (a) Differential expression profiles between BRCA1 and BRCA2 tumors. Using the immunohistochemical expression of the markers shown on the right, the majority of the BRCA1 (1) tumors were grouped in a separate cluster from the BRCA2 (2) tumors. Red indicates positive expression, green indicates negative expression, and the intensity of the color is a function of the immunohistochemical expression level. White indicates not valuable expression. (b) Cluster analysis of BRCA tumors and sporadic breast cancer cases (C). The group HER-2 positive (orange) of sporadic breast cancer is clustered in the in the ER negative branch separated from BRCA1 tumors.

considering that cyclin D1 is a protein induced by oestrogen, and its association with oestrogen receptor positivity has been clearly demonstrated in breast cancer. For the very first time our study identified cyclin D1 amplification in hereditary breast carcinomas. In *BRCA1* tumours the incidence of gene amplification (18%), was similar to that previously reported in sporadic cases (15–20%). In *BRCA2* tumours, although the frequency of this alteration was very high (60%), the low number of valuable cases precludes any conclusion. So, our data suggests a significant correlation between gene amplification and protein expression. In contrast, our FISH analysis showed that cyclin E amplification is a rare event in breast cancer [29, 30], including those occurring *BRCA1* and *BRCA2* mutation carriers. In fact, we did not find gene amplification

in any of the three groups confirming previous reports in sporadic cases.

Very little is known about the effect of cyclin D3 on any type of cancer. In a study on sporadic breast cancer by Wong et al. [31], an association between cyclin D3 and higher grade and presence of lymph node metastases was observed, an opposite pattern to that observed for cyclin D1. In our sample of hereditary and sporadic cases the expression of both cyclins was similar which is not surprising given their related functional activity. The expression of CDKs paralleled that of type D cyclins but was inverse to that of cyclins A in hereditary tumours. This is probably due to the fact that the CDKs p27 and p21 have a negative effect on cyclin A/CDK2 activity, but they seem to activate cyclin D/CDK complexes through at least three

Table 3. Comparison of immunohistochemical categorical variables and fish among familial IDCs with BRCA1 and BRCA2 mutation and sporadic cases

	BRCA1 n (%)	*p	BRCA2 n (%)	*p	Sporadic vs. BRCA1 n (%)	*p
Grade						
1	0/20		1/14(7.1)		6/57 (10.5)	
2	3/20 (15)		6/14 (42.9)		24/57 (42.1)	
3	17/20 (85)	NS(0.072)	7/14 (50)	NS	27/57 (47.4)	0.012
HER-2 (herceptest)						
Positive	0/20	NS	0/13	NS (0.066)	12/56 (21.4)	0.024
Adhesion molecules						
E-Cadherin						
Preserved	10/20 (50)	NS(0.092)	11/14 (78.6)	0.012	23/56 (41.1)	NS
P-Cadherin						
Present	4/20 (20)	NS	1/14 (7.1)	NS	1/57 (1.8)	0.004
β-Catenin						
Preserved	7/20 (35)	NS	6/14 (42.9)	NS	31/57 (54.4)	NS
γ-Catenin						
Preserved	6/19 (31.6)	NS	6/14 (42.9)	0.002	5/57 (8.8)	0.014
P120^{ctn}						
Preserved	5/19 (26.3)	NS	4/12 (33.3)	NS	29/56 (51.8)	NS(0.054)
Apoptosis						
BCLXL						
0	15/16 (93.8)		12/12 (100)		47/57 (82.5)	
3	1/16 (6.3)	NS	0/12	NS	10/57 (17.5)	NS
BAX						
0	1/15 (6.7)		1/12(8.3)		4/57 (7)	
1	7/15 (46.7)		2/12 (16.7)		10/57 (17.5)	
2	3/15 (20)		3/12 (25)		18/57 (31.6)	
3	4/15 (26.7)	NS	6/12 (50)	NS	25/57 (43.9)	NS
Survivin						
0	1/17 (5.9)		1/12(8.3)		0	
1	5/17 (29.4)		2/12 (16.7)		20/57 (17.5)	
2	1/17 (5.9)		3/12 (25)		9/57 (31.6)	
3	10/17 (58.8)	NS	6/12 (50)	NS (0.098)	28/57 (43.9)	NS
NFKB						
0	2/17 (11.8)		0/12		4/57 (7)	
1	6/17 (35.3)		1/12 (8.3)		20/57 (35.1)	
2	3/15 (17.6)		5/12 (41.7)		17/57 (29.8)	
3	6/15 (35.3)	NS	6/12 (50)	NS	16/57 (28.1)	NS
Fish						
HER-2						
Positive	0/15	NS	0/9	NS	12/54 (22.2)	0.045
c-MYC						
Positive	3/13 (23.1)	NS(0.087)	4/6 (66.6)	NS	19/53 (35.7)	NS
CCND1						
Positive	2/11 (18.2)	NS(0.094)	3/5 (60)	NS	20/56 (35.7)	NS
CCNE						
Positive	0/15	NS	0/8	NS	0/43	NS

*p (χ^2 test. Statistical signification). NS (not statistically significant).

different mechanisms: the assembly of cyclin D-CDKs complexes, increased nuclear localization of these complexes, and increased stability of D type cyclins [11]. The expression of p27 has been linked to the expression of ER and good prognosis in sporadic

breast cancer [32] while the association between p16 and p21 expression and clinicopathological features is more controversial [33–36].

According to their cell cycle regulatory defects, the phenotypes of *BRCA1* and *BRCA2* carcinomas are

similar to those proposed by Landberg [27] with a lower number of markers differentiating at least two subgroups of sporadic breast cancer. One of them, such as *BRCA2* carcinomas, was characterized by ER-positivity, high cyclin D1, and p27 and seemed to induce cell proliferation through a preserved Rb pathway. The second group, like *BRCA1* carcinomas, was ER-negative, and showed defects in p53 and p27. These tumors had a more substantial lack of G1/S control adopting and Rb-independent mechanism of cell proliferation [37]. Our analysis also suggested that *BRCA1* carcinomas showed additional alteration of the G2/M checkpoint, since they had increased expression of cyclin A. This alteration could also influence chromosome instability, which is a characteristic of *BRCA1* tumors [38].

In part, our cluster analysis supports this dualistic view of cell cycle alteration not only in breast cancer in general but also in hereditary, indicating that ER and p53 status might be the main determinant of cell cycle protein expression. The ER-positive/p53-negative phenotype, in which most *BRCA2* carcinoma were included, was associated with lower expression of Ki-67 and higher levels of the cell cycle proteins cyclin D1, cyclin D3, p27, p16, p21, CDK4, CDK2 and CDK1. On the other hand, the ER-negative/p53 positive phenotype, characteristic of most *BRCA1* carcinomas, showed an increased expression of Ki67 and the cell cycle molecules E2F6, cyclins A, B1 and E, SKP2 and Topo II α . A group of sporadic ER-negative/p53-negative/HER2-positive breast carcinomas seems to have an intermediate phenotype with respect to the expression of these cell cycle molecules

With regard to apoptotic markers, over expression of BCL2 was present in *BRCA2* confirming the good correlation between this markers and ER status. In contrast, high levels of caspase 3 were observed in *BRCA1* tumors. Caspase 3 is a cytosolic enzyme that is activated only in cells committed to undergo apoptosis and correlates strongly with morphological assessment. Thus, previous studies have shown that the apoptotic index obtained by measuring caspase activation was higher in high-grade, ER-negative tumors [39] as we observed in our group of *BRCA1*-associated carcinomas.

The expression of the basal cell markers, especially CK5/6, was significantly higher in *BRCA1* than in *BRCA2* and sporadic carcinomas. Some reports have established that breast carcinomas with expression of basal cell markers show specific characteristics in relation to their morphology, proliferation and prognosis [18]. Recently, Sorlie et al. [40], reanalyzing cDNA microarray data from van't Veer et al. [41], observed that most *BRCA1* carcinomas had a basal type gene expression profile. Similar results have been obtained by Foulkes et al. with immunological markers [42]. Then, although we have only analyzed two basal cell markers (P-cadherin and CK5/6), we have found that a high percentage of *BRCA1* tumors had the basal phenotype

while it was very infrequent in *BRCA2* (one case). This case was also ER/HER2-negative according to the basal phenotype pattern.

Overall, our analysis in hereditary breast cancer further defines the molecular differences between *BRCA1* and *BRCA2* tumors with respect to cell cycle, apoptosis and basal cell markers. Most *BRCA1* carcinomas were high grade, highly proliferating ER/HER2-negative breast carcinoma that frequently had a basal phenotype and were characterized by up-regulation of cyclin A and caspase 3, but downregulation of type D cyclins (D1 and D3), CDKs (p16, p21, p27), and BCL2. The opposite phenotype was found in most *BRCA2* carcinomas. Our study also demonstrates the potential of TMA analyses for the molecular classification of breast carcinomas into clinically and biologically relevant subgroups.

Acknowledgements

Supported by SAF03-02497, and SAF01-0075 grants. E.H. and D.S are recipients of research grants from the Fondo de Investigaciones Sanitarias, A.O. of a grant from the Comunidad de Madrid, and S.R. of a grant from the Fundación Inocente.

We thank to the Spanish National Tumor Bank Network and the Immunohistological Unit of the Centro Nacional Investigaciones Oncológicas for their assistance. We also thank Carmen Martin and Amanda Wren for their technical support.

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