Estrogen Receptor Status Could Modulate the Genomic Pattern in Familial and Sporadic Breast Cancer

Lorenzo Melchor,[1] Emiliano Honrado,[1] Jia Huang,[4] Sara Álvarez,[2] Tara L. Naylor,[4] María J. García,[1] Ana Osorio,[1] David Blesa,[2] Michael R. Stratton,[5] Barbara L. Weber,[4] Juan C. Cigudosa,[2] Nazneen Rahman,[5] Katherine L. Nathanson,[4] and Javier Benítez[1,3]

Abstract

Purpose: Familial breast cancer represents 5% to 10% of all breast tumors. Mutations in the two known major breast cancer susceptibility genes, *BRCA1* and *BRCA2*, account for a minority of familial breast cancer, whereas families without mutations in these genes (BRCAX group) account for 70% of familial breast cancer cases.

Experimental Design: To better characterize and define the genomic differences between the three classes of familial tumors and sporadic malignancies, we have analyzed 19 BRCA1, 24 BRCA2, and 31 BRCAX samples from familial breast cancer patients and 19 sporadic breast tumors using a 1-Mb resolution bacterial artificial chromosome array-based comparative genomic hybridization.

Results: We found that BRCA1/2 tumors showed a higher genomic instability than BRCAX and sporadic cancers. There were common genomic alterations present in all breast cancer groups, such as gains of 1q and 16p or losses of 8ptel-p12 and 16q. We found that the presence/absence of the estrogen receptor (ER) may play a crucial role in driving tumor development through distinct genomic pathways independently of the tumor type (sporadic or familial) and mutation status (BRCA1 or BRCA2). ER^- tumors presented higher genomic instability and different altered regions than ER^+ ones.

Conclusions: According to our results, the BRCA gene mutation status (mainly BRCA1) would contribute to the genomic profile of abnormalities by increasing or modulating the genome instability.

Two major genes associated with susceptibility to hereditary breast cancer have been identified to date: *BRCA1* and *BRCA2* (1, 2). Inheritance of a mutation in these genes confers an increased lifetime risk of breast cancer (60-85%) and ovarian cancers (15-40%; ref. 3), although these genes only explain ~25% of breast cancers within high-risk families (4, 5). Some groups have tried to find putative BRCAX gene(s) using linkage analysis to explain the genetic background of some of the remaining high-risk families but without conclusive results (6–9).

A large amount of data have been presented showing that breast tumors from patients with germ-line mutations in the *BRCA1* and *BRCA2* genes present morphologic and genetic differences and also differ from BRCAX tumors and sporadic breast cancer cases (see refs. 10, 11 for review). These data suggest that hereditary mutations in *BRCA1* and *BRCA2* lead to breast cancer development through different signaling pathways. One feature of solid tumors, and thus of breast cancer, is genomic instability, which involves chromosomal changes, such as DNA gains or losses. To understand these changes, many groups have pursued tumor genome profiling of different classes of breast cancer. By chromosomal comparative genomic hybridization (cCGH; ref. 12), *BRCA1*-associated tumors are characterized by a high frequency of losses of 5q, 4q, 4p, 2q, and 12q; whereas *BRCA2*-associated tumors present frequent losses of 13q and 6q and gains of 17q22-q24 (13). In addition, and because of the recent development of classifiers based on specific DNA copy number alterations of each tumor class (14–16), cCGH profiling has been proposed as a potential diagnostic tool.

More recently, array-based CGH (aCGH) has become widely used, providing higher resolution and flexibility than cCGH (see refs. 17–19 for review). The single analysis of familial breast cancer using aCGH published to date has confirmed

Authors' Affiliations: [1]Human Genetics Group and [2]Molecular Cytogenetics Group, Human Cancer Genetics Program, Spanish National Cancer Center (CNIO); [3]Center for Biomedical Research on Rare Diseases, Madrid, Spain; [4]Division of Medical Genetics, Department of Medicine, and Abramson Cancer Center, University of Pennsylvania, Philadelphia, Pennsylvania; and [5]Section of Cancer Genetics, Institute of Cancer Research, Sutton, Surrey, United Kingdom

Received 3/28/07; revised 7/5/07; accepted 9/14/07.

Grant support: Spanish Ministry of Education and Science grant FPU AP-2004-0448 (L. Melchor), ICRETT/05/063 and European Molecular Biology Organization Short-Term Fellowship ASTF 162-05, Spanish Ministry of Education and Science projects SAF-03-02497 and SAF-06-06149, and Breast Cancer Research Foundation (K.L. Nathanson).

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

Requests for reprints: Javier Benítez, Human Genetics Group, Human Cancer Genetics Program, Spanish National Cancer Center, 28029 Madrid, Spain. Phone: 34-91-224-69-55; Fax: 34-91-224-69-23; E-mail: jbenitez@cnio.es.

doi:10.1158/1078-0432.CCR-07-0711

some previous findings, such as the higher genomic instability and specific losses of 4p, 4q, and 5q observed in *BRCA1*-associated tumors and frequent gains of 17q24 found in breast cancers associated with *BRCA2* mutations (20). The authors also described a set of chromosomal regions that correctly discriminated among BRCA1, BRCA2, and sporadic breast tumors. However, BRCAX cases were not included in this study, and the results need to be confirmed in larger and independent series.

In the present study, we aimed to, first, establish the genomic profile of sporadic and BRCA1, BRCA2, and BRCAX familial breast cancers using a 1-Mb resolution bacterial artificial chromosome (BAC) array platform and, second, identify regions commonly altered in all groups and regions that are class specific. Finally, because the array platform we used was the same as the one implemented by Jönsson et al., we tried to validate the aberrant regions they proposed in our tumor sample set.

Materials and Methods

Patients and tumor samples. We collected formalin-fixed, paraffin-embedded (FFPE) breast tumor tissues from 74 patients. These patients were selected from families with at least three women affected with breast/ovarian cancer and at least one of them diagnosed before 50 years of age or from families with female breast/ovarian cancer and at least one case of male breast cancer. All cases were studied for mutations and large rearrangements in the BRCA genes using standard procedures (4, 21). Nineteen cases had mutations in the *BRCA1* gene, 24 patients presented mutations in the *BRCA2* gene, and 31 cases were negative for germ-line mutations in the BRCA genes and therefore denoted as BRCAX tumors. Eight of the breast cancers with *BRCA2* mutations were provided by the Breast Cancer Genetics Team, Institute of Cancer Research (Sutton, Surrey, United Kingdom). We also included a set of snap-frozen sporadic breast tumors from 19 unselected patients without a family history of breast cancer. All data on sporadic samples were provided by the University of Pennsylvania and included in a previous study (22). BRCA gene mutation status, age at diagnosis, type of carcinoma, histologic grade, and estrogen receptor (ER) status are provided in Supplementary Table S1. ER status was considered as positive when the percentage of stained cells was $\geq$10% in an immunohistochemistry analysis, as previously reported (23, 24).

DNA extraction. Genomic DNA from frozen tissue sections was extracted using proteinase K digestion followed by phenol/chloroform purification. DNA from FFPE tumors was isolated according to the S. DeVries/F. Waldman protocol[6] with minor modifications. Briefly, two 30-μm sections were obtained from FFPE tumors, treated with xylene, incubated in glycine-Tris-EDTA [100 mmol/L glycine, 10 mmol/L Tris (pH 8.0), 1 mmol/L EDTA] and sodium sulfocyanate (1 mol/L), and finally digested with proteinase K and purified with phenol chloroform. All sections were previously examined and dissected with a scalpel to ensure at least 70% content of tumor cells.

BAC array platform. CGH was done onto the "1-Mb array" platform developed at the University of Pennsylvania (25) and previously used in similar studies (20, 22). Briefly, the platform is composed of a set of 4,134 publicly available human BAC clones spaced at ~1-Mb resolution across the genome. BAC DNA was amplified using oligonucleotide-primed PCR primers. At least two replicates of each BAC clone were printed on each slide using a Lucidea Array Spotter (Amersham Biosciences) and a spotting solution of 50% DMSO (25).

Array comparative genomic hybridization. For hybridization, 1 μg of test DNA and 1 μg of sex-matched pooled normal human DNA (obtained from a set of 10 healthy female or male volunteers) were labeled with either Cy3-dCTP or Cy5-dCTP by random priming (BioPrime Labeling kit, Invitrogen). The differentially labeled DNA samples were pooled, mixed with 100 μg of human COT-1 DNA, dried down, and rehydrated in 50 μL of a formamide-based buffer (25). After denaturation (10 min at 75°C) and preannealing (30 min at 37°C), hybridization was allowed for 48 to 72 h at 37°C in a moist chamber on a slowly rocking platform followed by a series of posthybridization washes: 2× SSC and 0.1% SDS (15 min, room temperature), 2× SSC and 50% formamide, pH 7.0 (15 min, 45°C), 2× SSC and 0.1% SDS (30 min, 45°C), and 0.2× SSC (15 min, room temperature). Finally, arrays were scanned on a GenePix 4000B dual scanner (Axon Instruments). Both test and reference DNA were labeled with the opposite dye in a separate experiment ("dye swap") to account for differences in dye incorporation and provide additional data points for analysis.

Data analysis. Fluorescence data from hybridization images were processed and analyzed with GenePix Pro 5.0 (Axon Instruments) to obtain the $\log_2$ ratios (tumor/reference) of each slide. aCGH normalization was done using the DNMAD application (26), which also allowed us to merge and filter replicate clones on the same slide and in the dye-swap experiment. We filtered out inconsistent replicates (those with a $\log_2$ ratio distance to the median $\log_2$ ratio of the replicates >0.3) and those clones that did not have available data in >70% of the cases (65 of 93 samples).

Finally, we established categorized copy number values using the binary segmentation algorithm implemented in the InSilico CGH software (0, 1, or -1 indicating no change, gain, or loss, respectively; ref. 27). We defined genomic regions as a group of at least two consecutive clones showing the same categorized copy number value. High-level DNA amplifications were considered when segmentation level was four times the segmentation gain level.

For visualization purposes, we used CGHAnalyzer (25, 28) and CGHExplorer (29) softwares.

Standardization of an artifactual-copy number variation pattern. We observed a recurrent genomic pattern of artifactual aberrations in our breast cancer sample set (see Results), which was previously reported in normal samples analyzed by cCGH (30) and recently also observed by aCGH and named artifactual-copy number variation (Ar-CNV).[7] Briefly, this artifactual pattern generates abnormal ratios in certain chromosomal regions, such as 1p36, 2q37, 4p16, 6p21, 9q34, 11q13, and 12q13 (Fig. 1A), which can be erroneously interpreted as gains in the analysis. We also obtained this Ar-CNV pattern in a set of normal FFPE tissue DNA versus normal control DNA hybridizations. Kirchhoff et al. (30) previously described a nonrandom pattern of deviations in normal samples by cCGH and, subsequently, applied a standardization approach to increase the specificity and the sensitivity of the technique, obtaining a dramatic decrease in false-positive results. Therefore, to diminish the effect of the Ar-CNV in our set, a standardization approach was applied to every sample showing this artifactual pattern. The standardization was done by subtracting from each clone $\log_2$ ratio the median value for that clone in the set of normal FFPE tissue DNA versus normal control DNA hybridizations that also presented the Ar-CNV pattern. After the standardization, cases were analyzed again and the aCGH profiles did not show the artifactual pattern anymore (Fig. 1B). Log_2 ratios of each sample are shown in Supplementary Table S2.

Fluorescence* in situ *hybridization studies. To verify the standardization approach, we did a fluorescence *in situ* hybridization (FISH)

[6] Protocol available from http://cc.ucsf.edu/people/waldman/Protocols/paraffin.html.

[7] D. Blesa et al. Detection of a pattern of artifactual copy number variations that can induce to overestimate changes on genome profiling analysis, submitted for publication.

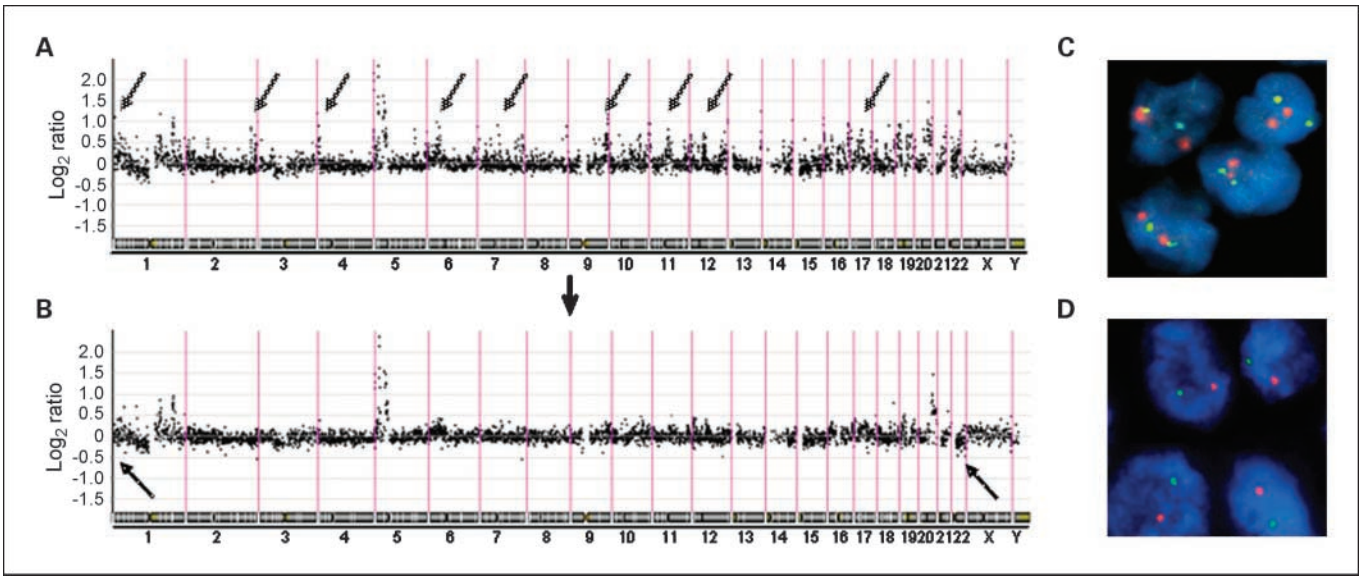

Fig. 1. Standardization of the Ar-CNV pattern. *A,* aCGH profile of a familial breast tumor sample that shows artifactual aberrations in the regions indicated by the arrows. *B,* aCGH profile of the same case after standardization. Aberrations at 1q, 5p, or 20q are considered gains because they are preserved after standardization. Arrows below the tumor profile indicate regions further validated by FISH analysis. *C,* FISH study using the 1p36 probe, showing two green signals (1p36 loci) and two red signals (centromeres). These data support the standardized aCGH pattern and not the nonstandardized one in which the gain was artifactual. *D,* FISH experiment with the chromosome 22 probe clearly shows a monosomy (one green and one red signal), supporting again the loss observed in the standardized profile.

analysis on tissue microarrays that contained the FFPE samples; their characteristics have been previously published (23, 24). We checked the copy number status of two different locations affected by Ar-CNV: 1p36 and 22q11-q12. The 1p36 probe was made of three BAC clones from the distal p-arm region of chromosome 1 (RP11-82D16, RP4-713A8, and RP4-740C4, located at 2.07, 2.25, and 2.30 Mb from the 1p telomere, respectively). These BACs were all labeled with dUTP-SpectrumGreen (Vysis, Inc.). The 1p36 probe also included a chromosome 1 centromeric probe "CEP1α Satellite DNA Spectrum-Orange" from Vysis. The 22q11-q12 probe consisted of two clones mapping at 22q11.21 (RP11-316L10 and RP11-330P17) labeled with dUTP-SpectrumGreen and three clones located on 22q12.2 (RP1-76B20, RP1-15I23, and RP3-394A18) labeled with dUTP-Spectrum-Orange. FISH analysis was done according to Vysis' instructions, with slight modifications. An average of 110 (50-200) well-defined nuclei was analyzed and the number of single copy gene and centromeric signals was scored.

Statistical analysis of aCGH data. We used a nonparametric Mann-Whitney *U* test to compare the mean number of genomic alterations among the four patient groups. The Statistical Package for the Social Sciences for Windows statistical software (SPSS, Inc.) was used for these comparisons. For the analysis of differences in the aberration frequency of specific chromosomal regions, we used the Stat POMELO tool[8] (31), applied Fisher's exact test, and adjusted *P* values for multiple testing using the false discovery rate approach (a *P* value of <0.05 was considered significant). Finally, hierarchical unsupervised clustering was done using correlation methods included in the Cluster software (32).

Results

Standardization procedure to diminish the effect of the Ar-CNV pattern. We analyzed tumor DNA from a total of 93 breast tumors (19 BRCA1, 24 BRCA2, 31 BRCAX, and 19 sporadic samples) using aCGH. We found a specific pattern of aberrations that was present in a high percentage of cases (78%). These genomic aberrations were coincident with the so-called Ar-CNV, described in a recent aCGH technical report. In this article, an aCGH pattern of specific Ar-CNV, observed in normal samples and distinct tumor types by using different array platforms and reproduced in several laboratories, is described.[7] We applied a standardization approach similar to the one used by Kirchhoff et al. (30) in their original cCGH studies to avoid an analogous issue (see Materials and Methods). An example of our standardization approach is shown in Fig. 1A and B. Some of the regions affected by Ar-CNV were further validated by FISH analysis (Fig. 1C and D; Supplementary Table S3). A closer correlation aCGH-FISH data was clearly found after standardization, showing a dramatic increase in confirmed aberrations (9% before to 82% after standardization). This analysis confirmed the utility of this correction.

Overall genomic changes in breast tumor classes. We determined the genome instability according to the number of CNVs (measured as the total number of gains, losses, and numerical aberrations) present in a tumor. *BRCA1*-associated tumors had the most unstable genome with a total of 28.0 ± 2.9 CNV, *BRCA2*-related tumors had 19.8 ± 2.3 CNV, BRCAX tumors showed 15.3 ± 1.9 CNV, whereas sporadic tumors presented 18.7 ± 1.9 CNV. These differences were statistically significant ($P < 0.05$, Mann-Whitney *U* test) when *BRCA1*-associated tumors were compared with the other tumor types (Supplementary Fig. S1A). No significant differences were found when a comparison between different types of recurrent mutations was made (data not shown).

Frequencies of genomic alterations. The frequency and distribution of genomic gains and losses of each group is shown in Fig. 2. Four genomic regions were altered in >40% of cases in all tumor groups: gains of 1q and 16p13.3 and losses of 8ptel-p12 and 16q. No specific aberrations were associated significantly with an exclusive breast tumor class. However, there were

[8] Statistics software available from http://pomelo.bioinfo.cnio.es.

some statistically significant differences in the pairwise comparisons (Table 1). Given that BRCAX tumors showed the lowest overall alteration frequency, many regions in BRCAX tumors were significantly different when compared with the other tumor classes (Table 1). Differences in the alteration frequencies in chromosome X were not considered because the reference for the sporadic breast cancer set was not sex matched.

There were aberrant regions at a high frequency (>50%) in each tumor class (Fig. 2). In summary, losses of 4q32.3-qtel, 5q, 13q, and 18q were more frequent in *BRCA1*-associated tumors. *BRCA2*-associated tumors presented recurrent gains of 8q12.3-qtel and losses of 11q23.1-qtel, 13q12.3-q21.33, and chromosome 22. Sporadic breast cancers had frequent gains of 8q13.1-qtel and losses of 4q24-qtel and 13cen-q31.3. Finally, BRCAX tumors did not show any highly recurrent aberration in addition to the commonly altered regions.

About high-level DNA amplifications, 8q24.11 and 8q24.12-q24.21 were the only two regions with a frequency >15% in all groups. No significant differences in high-level DNA amplification frequencies were found across tumor types. However, different tumor classes showed frequent high-level amplification (>15%) at distinct locations, such as 8p, 8q, 10p, 11q13, 12p13, and 13q34 (Table 1), pointing at subtle differences in the amplification targets of each tumor class. We looked in detail at specific regions of amplification that cover some known oncogenes, such as 8q24.21 (*c-MYC*) and 17q12 (*ERBB2*). *c-MYC* is amplified in all groups (37% BRCA1, 33% BRCA2, 26% sporadic, and 13% BRCAX). *ERBB2* was only amplified in 12.9% of BRCAX and 5.3% of sporadic cancers. Noteworthy, no high-level amplification of this gene was observed in *BRCA1*- and *BRCA2*-associated tumors.

Testing of the previously reported discriminative regions. To test the value of the discriminative regions described by Jönsson et al. (20), we used a hierarchical unsupervised clustering using the same discriminative chromosomal regions. We only included the mutation-positive BRCA carriers and sporadic breast cancer cases because these were the tumor classes previously used to describe these regions. We could distinguish two subgroups: one mainly composed of *BRCA1*-associated tumors (orange cluster) and other one mainly composed of *BRCA2*-associated and sporadic tumors (green cluster; Fig. 3A). However, we found BRCA2 (4) and sporadic (6) outlier tumors located in the BRCA1 subgroup and some BRCA1 samples (7) in the other branch. Jönsson et al. also found some outliers in their clustering. The authors suggested that ER status and grade might explain those outliers. When looking in detail at the ER status in our tumor series, we found that more than half the outliers in the BRCA1 branch (two BRCA2 and five sporadic tumors) were ER^-. Similarly, six of the seven BRCA1 outliers in the BRCA2 branch were ER^+ (Fig. 3A). This might indicate that these regions are mainly differentiating positive and negative ER tumors rather than BRCA mutation status.

Because Jönsson et al. only included in their study ER^- *BRCA1*-associated, ER^+ *BRCA2*-associated, and a mixture of $ER^{-/+}$ sporadic tumors, and we had a mixture of $ER^{-/+}$ in all tumor groups, we removed from our series all those cases that did not match the features of the series studied by Jönsson et al. (ER^+ *BRCA1*-associated and ER^- *BRCA2*-associated tumors). Without these tumors, we obtained a different cluster. The clustering analysis discriminated two groups, one of them (orange) mostly composed of ER^- tumors (17 ER^- and 2 ER^+ tumors) and a second branch (green) composed mainly of ER^+ tumors (22 ER^+ and 3 ER^- tumors; Fig. 3B). This time, the number of BRCA class outliers was reduced from 11 outliers (7 BRCA1 and 4 BRCA2; Fig. 3A) to 3 outliers (1 BRCA1 and 2 BRCA2; Fig. 3B). This cluster resembled the results obtained by Jönsson et al. These findings may highlight the importance of ER status in tumor development.

ER profiling of breast cancer. To further test the role of the ER, we divided all cases according to ER status, accounted for CNV in each group, and created genomic alteration frequency plots. The higher genomic instability in the ER^- tumors was remarkable, showing significant differences ($P < 0.05$) in CNV compared with the ER^+ tumor group (Supplementary Fig. S1B). We also saw this difference when we subdivided each tumor class according to ER status (Supplementary Fig. S1C).

Frequency plots of genomic alterations in the tumor groups according to the ER status are shown in Fig. 4A. Both groups of

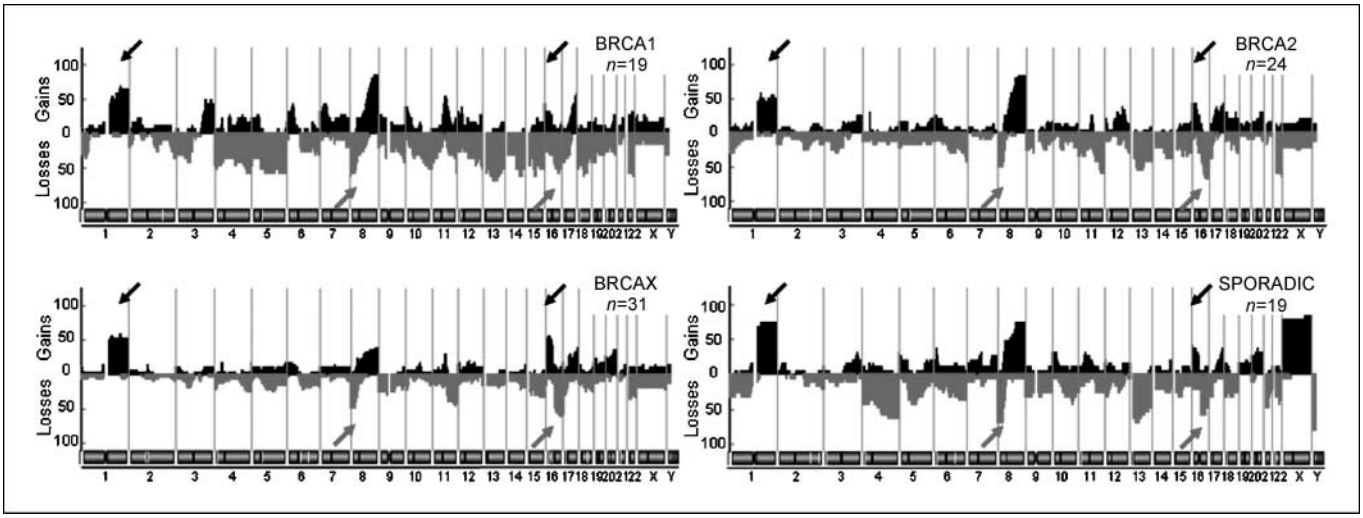

Fig. 2. Overall genomic aberration frequencies in BRCA1, BRCA2, BRCAX, and sporadic breast cancer samples. Black, frequency of gains; gray, frequency of losses. Arrows at chromosomes 1, 8, and 16 highlight the regions commonly altered in >40% of all breast cancer groups. Black arrows, common gains; gray arrows, common losses.

Table 1. Significant genomic aberrations (adjusted $P < 0.05$) and their frequencies among distinct breast tumor groups and recurrent high-level DNA amplifications

Chromosomal region	Frequencies of alteration (%)				Two-sided adjusted Fisher's exact *P*					
	BRCA1	BRCA2	BRCAX	Sporadic	B1vsB2	B1vsBX	B1vsS	B2vsBX	B2vsS	BXvsS
Gain										
8q22.1	**58**	**79**	32	**74**				*0.023*		
8q23.1-8q23.3	**74**	**83**	35	**74**				*0.010*		
8q23.3-8q24.13	**79**	**83**	35	**74**				*0.010*		
8q24.13-8qtel	**84**	**83**	39	**74**		0.077		*0.025*		
11q14.1	47	0	10	10	*0.005*					
Loss										
4q23	32	12	6	53						*0.020*
4q24	37	12	6	**58**						*0.005*
4q25	47	12	6	**58**		0.074				*0.005*
4q26-4q28.1	42	12	6	**58**						*0.005*
4q28.2	42	16	9	**58**						*0.031*
4q28.3	42	16	10	**63**						*0.022*
4q31.1	42	8	13	**63**					*0.009*	*0.022*
4q31.21	37	8	16	**63**					*0.009*	
4q32.1-4q32.2	**47**	12	23	63					*0.044*	
4q32.3	**52**	12	23	63					*0.044*	
4q33-4q34.1	**58**	12	26	63					*0.044*	
4q34.2-4q35.1	**58**	12	23	63					*0.044*	
4q35.1-4q35.2	**52**	12	26	63					*0.044*	
13q12.3	47	**50**	16	**68**						**0.013**
13q13.1-13q13.3	**53**	**50**	16	**68**						**0.013**
13q13.3-13q14.11	**58**	**54**	16	**68**						**0.013**
13q14.11-13q14.3	**63**	**54**	16	**68**		0.097				**0.013**
13q14.3-13q21.33	**68**	**54**	16	**58**		*0.019*				
13q22.1-13q22.2	**68**	42	16	**58**		*0.019*				
13q22.2-13q22.3	**68**	42	19	**58**		*0.048*				
13q31.1	**63**	42	19	**53**		*0.026*				
21q21.3	11	0	3	42					*0.025*	*0.046*
High-level DNA amplifications										
8p12-8p11.23	11	8	6	**16**						
8p11.22-8pcen	11	4	3	**16**						
8q22.1-8q22.3	5	**17**	10	**16**						
8q23.1-8q23.3	11	**21**	10	**21**						
8q24.11	**32**	**37**	**16**	**21**						
8q24.12-8q24.21	**37**	**37**	**16**	**21**						
8q24.21-8q24.22	**37**	**29**	13	**26**						
8q24.23-8qtel	**21**	**25**	10	**26**						
10ptel-10p15.3	0	0	0	**16**						
10p15.2	11	0	0	**16**						
10p15.1	0	0	0	**16**						
10p14	5	0	0	**16**						
11q13.3-11q13.4	11	12	**16**	0						
12p13.32-12p13.31	**16**	0	0	5						
13q34-13qtel	**16**	4	0	11						

NOTE: Frequency aberrations >50% are in bold. Entries in italics show $P < 0.05$. In addition, those high-level DNA amplifications with a frequency >15% (in bold) in at least one tumor group are shown.

tumors have common high frequent gains of 1q and 8q22.1-qtel and losses of 8ptel-p12 and 16q, which are similar to those described in all breast tumor classes. However, a set of genomic aberrations, such as -3p25.3-p21.3, -3p21.1-p14.2, +3q25.1-q26.1, -4q31.2-qtel, -5q, +10p14-p12.3, -12q14-q15, and -12q23.1-q23.31, presented significantly higher frequencies in ER^- tumors (Fig. 4B). It is noteworthy that 6q25.1 (the locus of the *ESR* gene) is more frequently deleted in ER^+ than in ER^- tumors.

In addition, ER^- tumors presented a trend toward having more high-level DNA amplifications than ER^+ tumors. There were also slight differences in the amplification target sites. For example, 8q24.21 (*c-MYC*) was amplified in 32% of the ER^- tumors and only in 20% of the ER^+ tumors. Moreover, 17q12 (*ERBB2*) was exclusively amplified in ER^- tumors (5%), whereas amplification at 20q13.12-q13.33 was only found in ER^+ tumors (8%). However, the only region with an amplification rate that was significantly different (adjusted *P* value < 0.05) comparing both groups was 13q34, which was only present in ER^- tumors (16%).

Discussion

The present aCGH-based study characterizes in detail the patterns of genomic alteration of the familial breast tumor classes (BRCA1, BRCA2, and BRCAX) and of sporadic breast

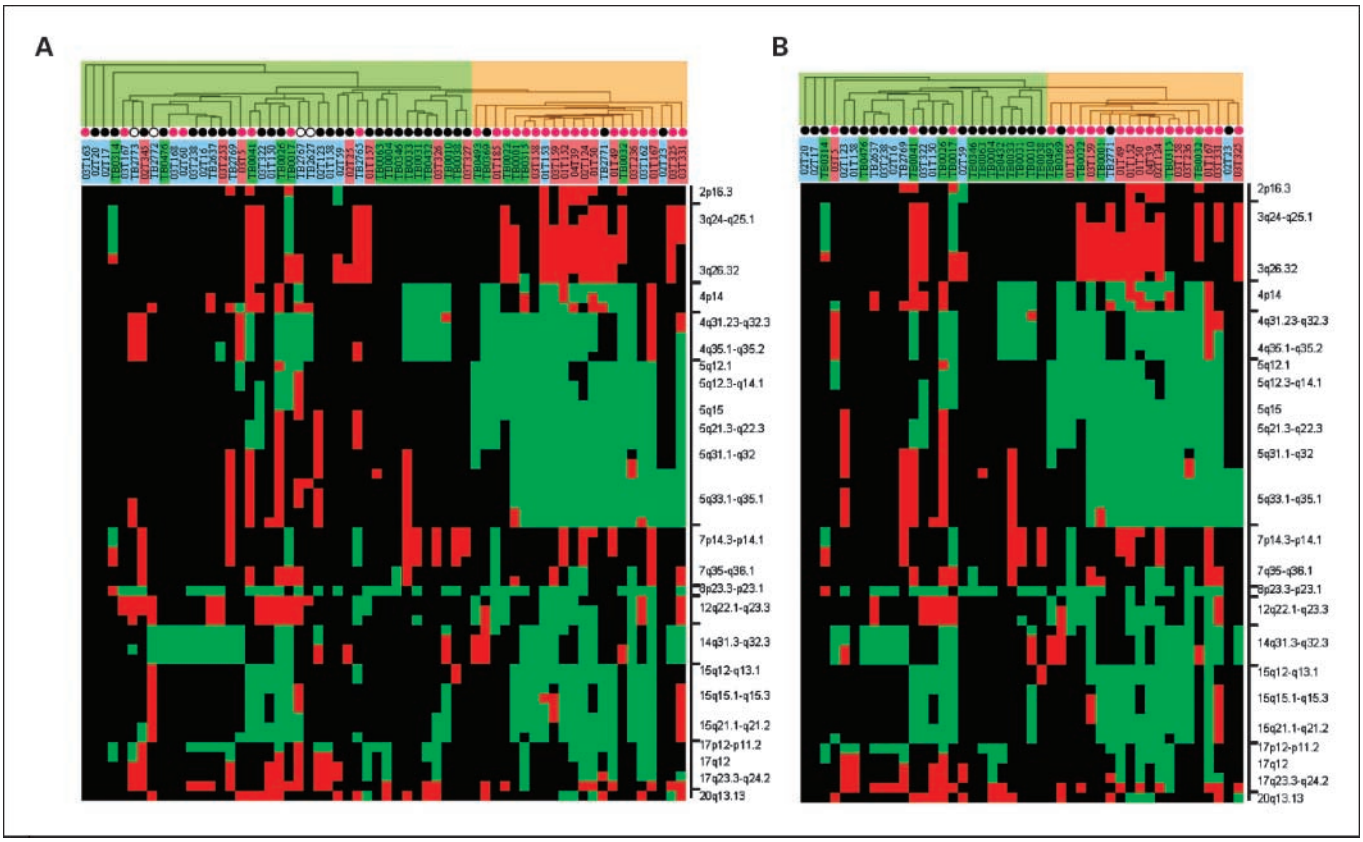

Fig. 3. *A,* unsupervised clustering of all BRCA1 (*red*), BRCA2 (*blue*), and sporadic (*green*) breast tumors of our collection using the regions reported as discriminative in the aCGH classifier by Jönsson et al. (20). Regions are listed according to chromosome number and are considered as gains (*red*), no change (*black*), or losses (*green*). Black circle, ER^+; red circle, ER^-; white circle, nonevaluated cases. *B,* unsupervised clustering of BRCA1 (*red*), BRCA2 (*blue*), and sporadic (*green*) breast tumors with BRCA1 ER^+ and BRCA2 ER^- tumors removed.

cancer, supports previous findings obtained using the cCGH technique and the same aCGH platform (20), and tests the discriminative regions defined in the latter study. To our knowledge, this is the first aCGH profiling of BRCAX-associated breast cancer samples and the second one of *BRCA1*- and *BRCA2*-associated tumors. Our results highlight the importance of ER status, suggesting that this feature should be considered when designing comparative studies of familial and sporadic breast cancer.

Genomic instability in familial and sporadic breast cancer. We have confirmed the high genomic instability in *BRCA1/2*-associated tumors that was previously observed in several articles using cCGH (13–16) and aCGH (20). The highest number of CNV was present in breast cancers associated with *BRCA1*, those related to *BRCA2* mutations were the second most unstable class of breast tumors, whereas sporadic cases showed more CNV than BRCAX-related breast cancers but less than those associated with *BRCA2* mutations (Supplementary Fig. S1A).

We distinguished common aberrations across all breast cancer groups, such as gains of 1q and 16p13.3 and losses of 8ptel-p12 and 16q, which are concordant with previous analyses by cCGH (15, 16, 33). These commonly altered regions could represent a set of shared aberrations that include important genes and characterize the breast cancer development in general.

Beyond these shared alterations, there were no specific genomic changes associated with any specific tumor class, although some recurrent aberrations were observed (Fig. 2). Thus, *BRCA1*-associated tumors were characterized by a high frequency of gains of 3q and 8q21.3-qtel and losses of 4q32.3-qtel and distinct regions of chromosome 5. *BRCA1*-associated tumors also had a significantly recurrent gain at 11q14.1 when compared with *BRCA2*-associated tumors (Fig. 1; Table 1). Some of these changes have been reported as discriminative in tumor class comparisons (13, 15, 20). On the other hand, *BRCA2*-associated tumors had frequent gains of 8q12.3-qtel (significant when compared with BRCAX) and losses of 11q23.1-qtel, 13q12.3-q21.33, and chromosome 22 (Fig. 1; Table 1). Gain of 17q22-q24 and losses of chromosome 13 among others have been reported as frequent changes in *BRCA2*-associated tumors (13, 20). Here, we found both changes at a high frequency, and we were also able to refine both changes to 17q23-qtel and 13q12.3-q21.33. BRCAX tumors were characterized by the lowest overall frequency of genomic alterations, presumably due to the high number of grade 1 samples in the BRCAX-associated breast cancer set (14 of 31; see Supplementary Table S1). Finally, regions recurrently aberrant in sporadic samples were concordant with the ones previously reported (34, 35).

In addition, amplification target sites slightly differed between tumor classes (Table 1). Interestingly, whereas c-*MYC* is amplified in all groups suggesting an universal amplification target site, neither *BRCA1*- nor *BRCA2*-associated tumors presented amplification at the *ERBB2* locus, as previously

described (10, 20). *ERBB2* amplification seems to be a marker that can help in discriminating familial samples, in which mutation analysis of the BRCA genes is advisable, saving time and effort.

With these results, the aCGH patterns of familial breast cancer may assist in the diagnostics because there are differences between BRCA1 and BRCA2/BRCAX patients. However, our findings show some differences when compared with previous studies about the discriminative regions associated with familial and sporadic cancer. These variations are largely attributable to differences in sample size or the type of *BRCA1/2* mutation of the set of familial cancer samples, but we propose an additional role of ER status. Whereas previous studies mainly studied BRCA1/ER$^-$ and BRCA2/ER$^+$ tumors, we were able to study a set of *BRCA1*- and *BRCA2*-associated breast cancers that contained both ER$^+$ and ER$^-$ tumors.

ER status modulates the genomic changes in the tumor. Steroid receptor status is one of the main differentiating features of sporadic breast cancer, as has been shown in gene expression studies (36) and in aCGH profiles (34, 35, 37). With regard to familial breast cancer, *BRCA1*-associated tumors mainly are associated with ER negativity, and for this reason, most of studies on BRCA1 tumors only include ER$^-$ *BRCA1*-associated breast cancer samples. Examples are the study by Hedenfalk et al. (38) using expression arrays and more recently the study by Jönsson et al. (20) using aCGH. It has been suggested that the ER status may have confounded the findings in these studies (39, 40). When we examined our series using Jönsson's discriminative regions, we found one cluster mainly composed of *BRCA1*-associated cases and another one consisting of *BRCA2*-associated and sporadic cases, although many outliers were present (Fig. 3A). The reason could be that

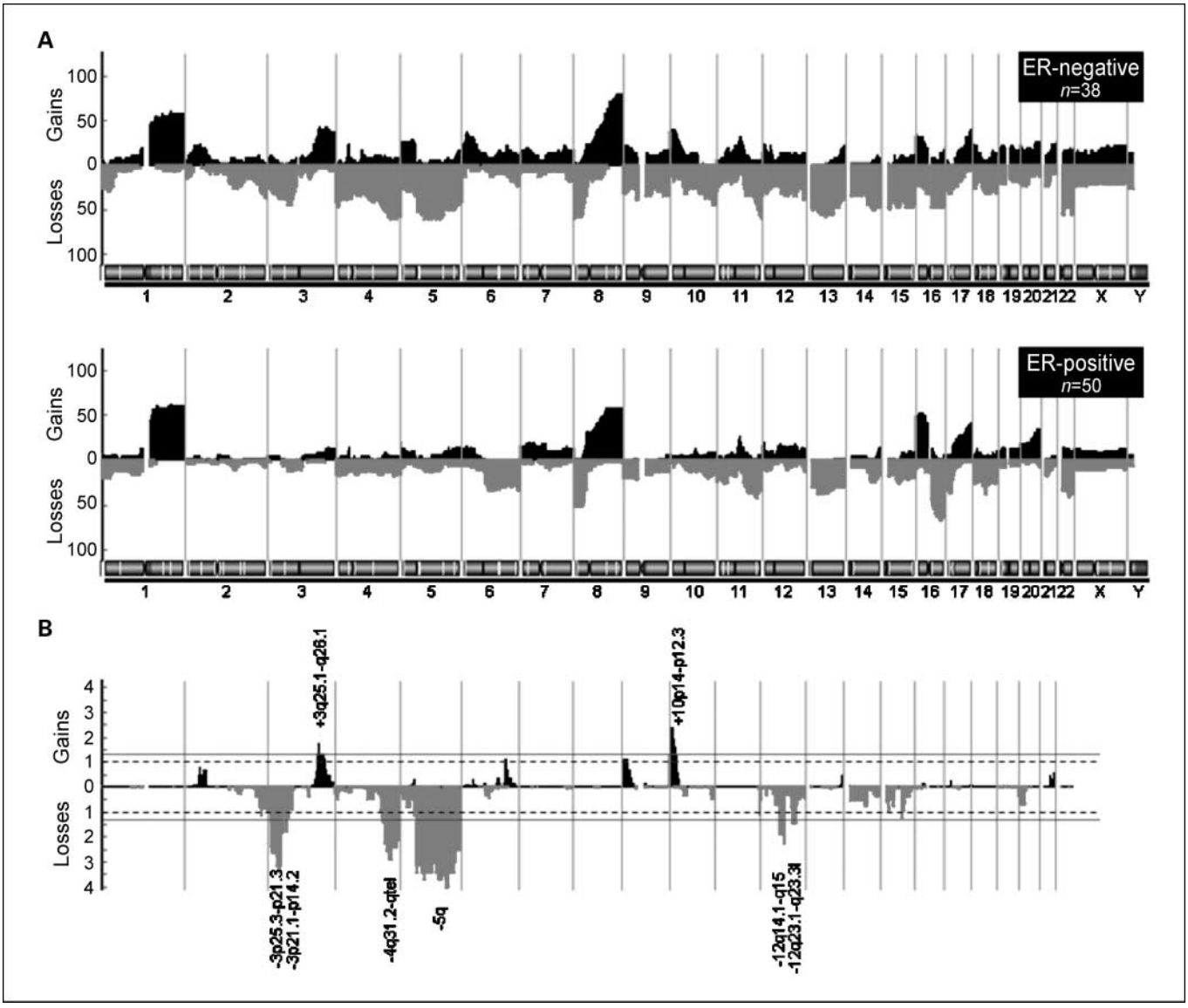

Fig. 4. *A,* overall genomic aberration frequencies of all tumors classified according to ER status. Black, frequency of gains; gray, frequency of losses. *B,* representation of the negative log of the adjusted *P* values, obtained from Fisher's exact test when comparing the gain (*top*) and loss (*bottom*) frequencies among ER$^-$ versus ER$^+$ tumors. Dashed lines, adjusted *P* value = 0.1 [-log (adjusted *P*) = 1]; gray lines, adjusted *P* value = 0.05 [-log (adjusted *P*) = 1.3]. Those aberrations that are statistically significant (*P* = 0.05, Fisher's exact test) are shown.

Jönsson's discriminative regions were found only comparing BRCA1 ER^-, BRCA2 ER^+, and sporadic tumors with either status, whereas our series included BRCA1 and BRCA2 tumors with a mix of ER^- and ER^+ status. In fact, after removing BRCA1 ER^+ and BRCA2 ER^- tumors from our series, we obtained one cluster composed of ER^- tumors (BRCA1 and sporadic) and a second cluster mainly formed by ER^+ tumors (BRCA2 and sporadic), the number of BRCA outliers being clearly reduced (Fig. 3B). Both ER clusters present obvious genomic differences from each other. These results suggest that ER status is an important marker of changes in the tumor genome, independent of the underlying mutation status.

When we grouped all tumors, including BRCAX samples, according to their ER status, the higher genomic instability of ER^- tumors was clearly shown not only when all cases were grouped together (Supplementary Fig. S1B) but also within each group defined by BRCA mutation status (Supplementary Fig. S1C). Several genomic aberrations were recurrently present in ER^- tumors, such as -3p25.3-p21.3, -3p21.1-p14.2, +3q25.1-q26.1, -4q31.2-qtel, -5q, +10p14-p12.3, -12q14-q15, and -12q23.1-q23.31, which significantly discriminated between both ER tumor groups (Fig. 4B). Most of these aberrations are concordant with those previously reported in aCGH studies of sporadic breast cancer (34, 35, 37), and some were also included in Jönsson's discriminative regions (20). In fact, losses of loci at 4q and 5q were reported as frequent in *BRCA1*-related breast cancers but also in ER^- *BRCA2*-associated breast tumors and ER^- sporadic samples (41, 42). On the other hand, ER^+ tumors showed a lower level of genomic instability and a trend to present alterations at chromosome 16 (+16p and -16q), which are classic features of ER^+ and low-grade tumors (35, 43, 44). Interestingly, absence of expression of ER seems to be due to a mechanism independent of copy number losses at the *ESR* locus, given that ER^+ tumors presented a higher deletion frequency at this locus than ER^- tumors (Fig. 4A). About high-level DNA amplifications, ER^- tumors had higher frequencies of these aberrations than ER^+ tumors. We propose here the significantly recurrent amplification at 13q34 in ER^- tumors as a candidate aberration for further characterization. The same differences between both tumor groups (ER^+/ER^-) about genomic instability and aberrant regions were also present when tumors were subdivided by BRCA mutation status (Supplementary Fig. S2), which emphasizes the role of the ER status independent of the breast tumor class. In this sense, comparisons between tumor classes with the same ER status could elucidate aberrations specifically associated with a breast tumor class. However, a larger amount of samples would be needed.

In summary, we present a genomic characterization of familial and sporadic breast cancer samples using aCGH. We confirm the higher genomic instability of BRCA1/2 tumors and describe the common existence of aberrations that could represent the set of genomic abnormalities characteristic in breast cancer development. We also report a set of altered regions that discriminate between tumor groups but are not specific for only one tumor class. Our findings suggest a critical role for the ER status as a marker of the genomic changes present in a tumor. The patterns of genomic changes among familial and sporadic breast cancers are strikingly similar, and differences are mainly determined by the ER status rather than by the BRCA mutation status as is generally accepted. Thus, ER negativity might allow or involve a set of genomic alterations different from those in tumors expressing ER. These aberrant regions may contain interesting genes that determine the higher aggressiveness of the ER^- tumors and, hence, should be analyzed in detail, whereas the BRCA gene mutation status (mainly BRCA1) would contribute to the genomic profile of abnormalities by increasing or modulating the genomic instability.

Acknowledgments

We thank the Spanish National Tumor Bank Network and Luz Álvarez and Miguel Urioste (Familial Cancer Unit) for the collection of the samples, the Immunohistological Unit and Carmen Martin (Molecular Cytogenetics Group, Spanish National Cancer Center) for their technical support, and Angelica C. Medina (Medical Genetics Division, University of Pennsylvania) for her technical assistance.

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Clin Cancer Res 2007;13:7305-7313.