

## Promoter Hypermethylation and BRCA1 Inactivation in Sporadic Breast and Ovarian Tumors

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**Background:** Inherited mutations in the BRCA1 gene may be responsible for almost half of inherited breast carcinomas. However, somatic (acquired) mutations in BRCA1 have not been reported, despite frequent loss of heterozygosity (LOH or loss of one copy of the gene) at the BRCA1 locus and loss of BRCA1 protein in tumors. To address whether BRCA1 may be inactivated by pathways other than mutations in sporadic tumors, we analyzed the role of hypermethylation of the gene's promoter region. **Methods:** Methylation patterns in the BRCA1 promoter were assessed in breast cancer cell lines, xenografts, and 215 primary breast and ovarian carcinomas by methylation-specific polymerase chain reaction (PCR). BRCA1 RNA expression was determined in cell lines and seven xenografts by reverse transcription-PCR. *P* values are two-sided. **Results:** The BRCA1 promoter was found to be unmethylated in all normal tissues and cancer cell lines tested. However, BRCA1 promoter hypermethylation was present in two breast cancer xenografts, both of which had loss of the BRCA1 transcript. BRCA1 promoter hypermethylation was present in 11 (13%) of 84 unselected primary breast carcinomas. BRCA1 methylation was strikingly associated with the medullary (67% methylated; *P* = .0002 versus ductal) and mucinous (55% methylated; *P* = .0033 versus ductal) subtypes, which are overrepresented in BRCA1 families. In a second series of 66 ductal breast tumors informative for LOH, nine (20%) of 45 tumors with LOH had BRCA1 hypermethylation, while one (5%) of 21

without LOH was methylated (*P* = .15). In ovarian neoplasms, BRCA1 methylation was found only in tumors with LOH, four (31%) of 13 versus none of 18 without LOH (*P* = .02). The BRCA1 promoter was unmethylated in other tumor types. **Conclusion:** Silencing of the BRCA1 gene by promoter hypermethylation occurs in primary breast and ovarian carcinomas, especially in the presence of LOH and in specific histopathologic subgroups. These findings support a role for this tumor suppressor gene in sporadic breast and ovarian tumorigenesis. [J Natl Cancer Inst 2000;92:564-9]

An unexpected finding in the human molecular genetics of cancer is the absence of somatic mutations of the breast cancer susceptibility gene BRCA1 in sporadic cases of breast carcinoma. BRCA1 was first mapped to chromosome 17q21 by linkage studies (1) and later isolated by positional cloning (2). Since the gene's cloning, germline mutations in BRCA1 have been found in the hereditary cases of breast and ovarian cancers (3,4). In fact, germline alterations in BRCA1 have been estimated to be responsible for about 50% of familial breast cancer (3,5). However, despite an extensive search, the BRCA1 gene had not been shown to be mutated in any cases of truly sporadic breast cancer and in only an extreme minority of sporadic ovarian tumors (6,7). These findings challenge the role of BRCA1 as a tumor suppressor gene in the nonhereditary forms of breast and ovarian neoplasia that constitute 90%–95% of these tumor types. However, two lines of evidence continue to support BRCA1 loss of function as an important contributor to breast and ovarian tumorigenesis in the nonfamilial cases. First, a high rate of loss of heterozygosity (LOH), an allelic deletion that usually pinpoints the presence of a tumor suppressor gene, has been observed at the BRCA1 locus in approximately one half of sporadic breast and ovarian carcinomas (8–10). Second, the BRCA1 transcript (11) and protein (12) are often decreased or lost in sporadic breast carcinomas. However, it remains uncertain which mechanisms, apart from LOH, are behind the above-mentioned loss of function of BRCA1 in the nonfamilial breast and ovarian tumors.

An alternative mechanism to intragenic mutations for the inactivation of tumor suppressor genes is promoter hyper-

methylation (13,14). Methylation is the main epigenetic modification in humans (13), and changes in patterns of methylation play an important role in tumorigenesis. In particular, hypermethylation of normally unmethylated CpG islands located in the promoter regions of many tumor suppressor and DNA repair genes, such as p16, p15, Rb, VHL, E-cadherin, GSTP1, MGMT, and hMLH1, is associated with its loss of expression in cancer cell lines and primary tumors (13–18). In the cases of p16 and hMLH1, germline point mutations are responsible for genetic diseases carrying an increased risk of melanoma and of colorectal, endometrial, and gastric malignancies, respectively, but somatic mutations of these genes are infrequent in sporadic tumors. In these nonfamilial malignancies, silencing of p16 and hMLH1 by promoter methylation is often the most frequent alteration (13,18,19).

Several reports (20–24) suggest that aberrant methylation of BRCA1 could occur in breast carcinoma. This change has been observed in some breast cancer cell lines and primary tumors. However, a relationship among this methylation, BRCA1 gene expression, and incidence of LOH has not been reported. To assess whether BRCA1 may be inactivated by epigenetic mechanisms, we have studied 215 primary breast and ovarian tumors and a series of breast cancer cell lines and xenografts for hypermethylation affecting the CpG island located in the 5' region near the main transcription start site in the BRCA1 gene. Our results suggest that epigenetic loss of BRCA1 function by promoter hypermethylation, associated

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with LOH and particular histologic subtypes, occurs frequently in sporadic primary breast and ovarian carcinomas.

## MATERIALS AND METHODS

**Study subjects.** The 194 primary breast carcinomas were consecutive surgical resections collected from The Johns Hopkins Hospital (Baltimore, MD), the Clinica Puerta de Hierro (Madrid, Spain), and the Hospital Santa Cristina (Madrid). The 21 primary ovarian carcinomas were collected from Hospital de la Santa Creu i Sant Pau (Barcelona, Spain). The protocol for tissue collection was approved by each institution's review board. Tumors and corresponding normal tissues were snap-frozen in liquid nitrogen immediately after resection. All specimens underwent histologic examination by two pathologists 1) to confirm the diagnosis of adenocarcinoma, 2) to confirm the presence of tumor, and 3) to evaluate the percentage of tumor cells constituting these samples.

**Analysis of LOH at the BRCA1 locus in primary breast and ovarian tumors.** All samples consisted of at least 75% tumor cells. DNA was extracted from paired normal and tumor samples with the use of a nonorganic method (Oncor, Inc., Gaithersburg, MD). Polymerase chain reaction (PCR) was performed in 25- $\mu$ L volumes with the use of 0.2 U of *Taq* DNA polymerase and 1  $\times$  PCR buffer (Promega Corp., Madison WI), 200  $\mu$ M deoxynucleoside triphosphate, 30 pmol of each primer, and different concentrations of KCl and MgCl<sub>2</sub>, depending on the polymorphic marker. A 30-cycle amplification was done in a thermal cycler (The Perkin-Elmer Corp., Foster City, CA). For the breast samples, two polymorphic markers of the 17q21 region and intragenic to BRCA1 gene were used, D17S855 and D17S1323 (provided by D. Goldgar, University of Utah Medical Center, Salt Lake City). Sixty-six (82%) of 80 of the breast tumors examined in this series were informative at one of two markers: D17S855 was informative in 60% of the cases, and D17S1323 was informative in 62%. The 66 informative tumors include 25 previously reported (25) and 41 newly analyzed tumors. For the ovarian samples, the markers were D17S250, TRHA1, D17S800, D17S855, and D17S579. The alleles were separated by mixing 25  $\mu$ L of the PCR products with a 10- $\mu$ L volume of loading buffer (total volume, 35  $\mu$ L), 0.02% xylene cyanol, and 0.02% bromophenol blue. Electrophoresis was run on non-denaturing 8%–12% polyacrylamide gels for 12–15 hours at 500 V. After gel electrophoresis, the allelic band intensity was detected by a nonradioisotopic technique by use of a commercially available silver-staining method (26). Allele intensity was analyzed by densitometry. The gel image was captured by use of a GS-690 Imaging Densitometer (Bio-Rad Laboratories, Hercules, CA), digitized in 400 dots per inch, and the image analyzed by use of a Multi-Analyst/PC (Bio-Rad Laboratories). An allele was considered to be lost when its signal was reduced by more than 50% with respect to that observed on the normal counterpart DNA. Concerning the 21 breast cancer cell lines and xenografts, all of the cell lines were obtained from the American Type Culture Collection (Manassas, VA), except MPE600 (provided by Dr. Helene Smith, California Pacific Medical Center, San Francisco) (27), while the origin of

the xenografts (breast tumors grown in immunodeficient [SCID] mice) has been previously described (28).

**Analysis of BRCA1 promoter methylation patterns.** DNA methylation patterns in the CpG islands of BRCA1 gene were determined by methylation-specific PCR (29) in primary tumors and cell lines. The breast cancer cell lines used in the study were BT20, BT474, BT549, CAMA-1, DU4475, EVSA-T, Hs578T, MCF-7, MPE600, MDA-MB-157, MDA-MB-175VII, MDA-MB-231, MDA-MB-361, MDA-MB-435S, MDA-MB-453, MDA-MB-468, SK-BR-3, T47D, UACC-812, UACC-893, and ZR75-1. Methylation-specific PCR distinguishes unmethylated from methylated alleles in a given gene on the basis of sequence changes produced following bisulfite treatment of DNA, which converts unmethylated, but not methylated, cytosines to uracil and subsequent PCR by use of primers designed for either methylated or unmethylated DNA (28). Primer sequences of BRCA1 for the unmethylated reaction were 5'-TTG GTT TTT GTG GTA ATG GAA AAG TGT-3' (sense) and 5'-CAA AAA ATC TCA ACA AAC TCA CAC CA-3' (antisense) and for the methylated reaction 5'-TCG TGG TAA CGG AAA AGC GC-3' (sense) and 5'-AAA TCT CAA CGA ACT CAC GCC G-3' (antisense). The sense primer of the unmethylated reaction begins at 1536 base pairs (bp), and the sense primer of the methylated reaction begins at 1543 bp from GenBank sequence U37574. The unmethylated product is 86 bp long, and the methylated product is 75 bp. This region crosses the major transcription start site at 1581 bp (30). Placental DNA treated *in vitro* with *Sss*I bacterial methylase was used as a positive control for methylated alleles of BRCA1. DNA from normal lymphocytes was used as a negative control for methylated genes. Ten microliters of each PCR reaction was loaded directly onto nondenaturing 6% polyacrylamide gels, stained with ethidium bromide, and visualized under UV illumination.

**Reverse transcription (RT)-PCR of BRCA1.** Isolation of total RNA by the use of the Rneasy kit by Qiagen, Valencia, CA) and preparation of complementary DNA (cDNA) with the use of Ready-to-go-you-prime-first-strand beads of Amersham Life Science Inc. (Arlington Heights, IL)/Pharmacia Biotech, Inc. (Piscataway, NJ) was done according to the manufacturer's instructions. Briefly, 5  $\mu$ g of total cellular RNA and gene-specific forward and reverse primers were added to the beads. The cDNA reaction was run for 1 hour at 37 °C. PCR conditions were a hot start of 4 minutes at 94 °C and then 30 cycles of 30 seconds at 94 °C, 1 minute at 54 °C, and 1 minute at 72 °C, followed by a final extension of 5 minutes at 72 °C. Two primer sets for BRCA1 were used: 5'-TGG AAG AAA GTG AAC TTG ATG-3' (sense) plus 5'-CCT CTG AAC TGA GAT GAT AG-3' (antisense) and 5'-ATG CTG AAT GAG CAT GAT TTT G-3' (sense) plus 5'-AGA GTG CTA CAC TGT CCA AC-3' (antisense), this last set spanning several exons to avoid amplification of DNA. RT-PCR for hypoxanthine phosphoribosyltransferase by use of the primers 5'-GTG GGG TCC TTT TCA CCA G-3' (sense) and 5'-TAT GGA CAG GAC TGA ACG TC-3', again spanning several exons to ensure that only RNA was amplified, served as a positive internal control. Ten microliters from each PCR reaction was directly loaded onto agarose gel, stained

with ethidium bromide, and visualized under UV illumination.

**Statistical analysis.** All comparisons for statistical significance were performed by use of  $\chi^2$  and Fisher's exact test, as appropriate, with all *P* values representing two-tailed tests and statistically significant at .05.

## RESULTS

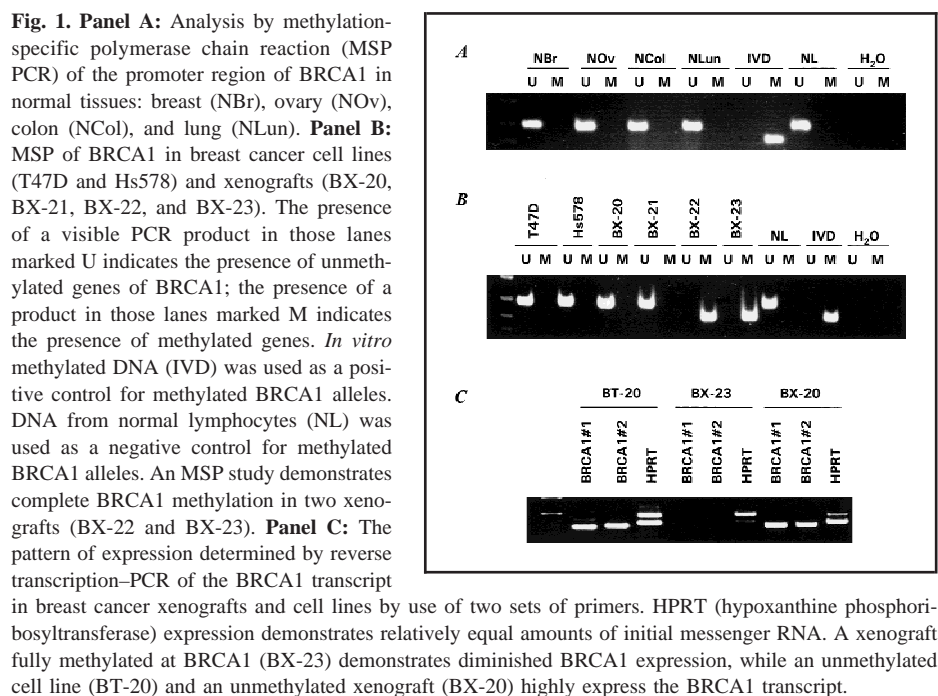
### BRCA1 Promoter Hypermethylation and Expression in Breast Cancer Cell Lines and Xenografts

DNA samples from normal lymphocytes, breast, ovary, lung, colon, and liver were completely unmethylated at the BRCA1 promoter (Fig. 1, A), the pattern normally observed for a CpG island. None of our 21 breast cancer cell lines had abnormal BRCA1 methylation (Fig. 1, B). However, when the BRCA1 promoter hypermethylation was studied in seven breast cancer xenografts in immunodeficient mice, two of them demonstrated complete methylation at this region (Fig. 1, B). Both methylated xenografts also had LOH at the BRCA1 locus.

Expression of BRCA1 determined by RT-PCR revealed that the six unmethylated breast cancer cell lines and an unmethylated breast cancer xenograft expressed the BRCA1 transcript (Fig. 1, C). However, in the two breast cancer xenografts completely methylated at the BRCA1 promoter, the expression of the BRCA1 transcript was abolished (Fig. 1, C).

### BRCA1 Promoter Hypermethylation in Primary Breast and Ovarian Carcinomas

To address the relevance of the promoter hypermethylation of the BRCA1 gene *in vivo*, we examined 84 unselected primary breast carcinomas. Hypermethylation of the BRCA1 promoter was detected in 11 (13%) of the 84 tumors examined (Fig. 2, A). The presence of abnormal BRCA1 methylation was more common in breast tumors from patients less than or equal to 45 years old (19% versus 4%) but did not reach statistical significance (*P* = .056; two-tailed Fisher's exact test). BRCA1 methylation was also more common when the tumor size was greater than 2 cm (seven of 42 tumors versus one of 34; *P* = .068 [tumor size was unavailable for the remaining eight]). In the 73 invasive ductal carcinomas, BRCA1 methylation was observed only in the grade 2 or 3 tumors. In this group of



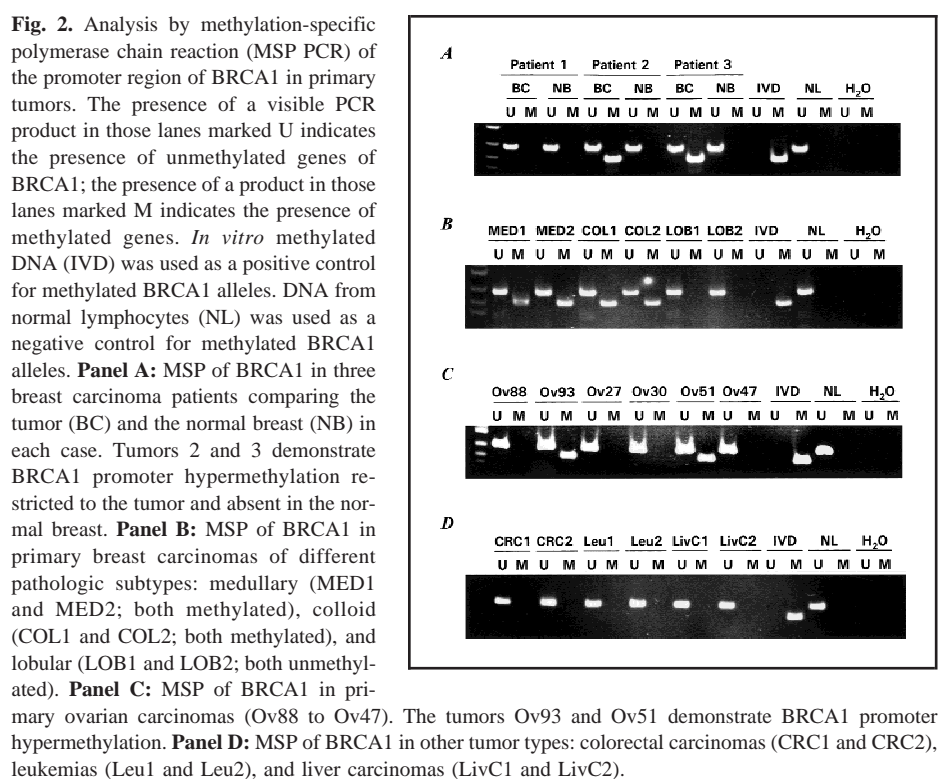
tumors, there was no association of BRCA1 methylation with the presence of lymph node metastasis ( $P = .72$ ), estrogen receptor status ( $P = .47$ ), or progesterone receptor status ( $P = .44$ ). The presence of BRCA1 methylation was not related to the abnormal methylation of GSTP1 ( $P = 1.00$ ), which had been previously demonstrated in a subset of these tumors (15).

Two particular histologic subtypes of breast carcinoma, medullary and mucinous carcinomas, appear on the basis of earlier studies (31–33) to be overrepresented in BRCA1-inherited breast tumors. Among the unselected sporadic breast tumors described above, we found that, while most (nine of 11) of the tumors with methylation were of ductal origin, the other two methylated tumors consisted of

one mucinous and one medullary carcinoma. Therefore, we studied BRCA1 methylation in an additional set of 34 sporadic tumors that were selected on the basis of these and other histologic subtypes (also lobular and papillary), all of which are uncommon compared with ductal. When the unselected and selected groups were combined, BRCA1 promoter hypermethylation was present in six (55%) of 11 sporadic mucinous breast carcinomas ( $P = .0033$ ; mucinous versus ductal) and in eight (67%) of 12 medullary breast carcinomas ( $P = .0002$ ; medullary versus ductal) (Fig. 2, B; Table 1). The higher rates of methylation obtained in these subtypes suggest an important role of the BRCA1 inactivation in the development of breast carcinomas with mucinous and medullary features. Supporting these data, a further analysis of two other subtypes of breast carcinomas uncommon in the familial cases associated with BRCA1, lobular and papillary carcinoma, demonstrated that none of 11 primary breast lobular carcinomas and none of 11 primary papillary breast carcinomas had BRCA1 promoter hypermethylation (Fig. 2, B; Table 1). Thus, epigenetic inactivation of BRCA1 in the sporadic tumors resembles the patterns described in the BRCA1 families with inherited genetic defects.

To determine whether BRCA1 promoter hypermethylation is associated with the frequent loss of chromosomal material at one allele of BRCA1 observed in breast carcinoma, we examined 66 additional primary invasive ductal carcinomas that had, or were without, LOH at the BRCA1 locus. This group of tumors, informative for markers within the BRCA1 gene, represents 82% of the invasive ductal tumors examined. Aberrant methylation of the BRCA1 promoter was found in nine (20%) of 45 breast carcinomas with LOH but was present in only one (5%) of 21 tumors without LOH ( $P = .15$ ; Fisher's exact test) (Table 1).

We next analyzed BRCA1 hypermethylation in 31 primary ovarian carcinomas, the other tumor site clearly associated with BRCA1 germline mutations, studying only tumors informative for LOH at the BRCA1 locus. BRCA1 promoter hypermethylation was present in four (31%) of 13 sporadic ovarian carcinomas with LOH at BRCA1, while none of the 18 primary ovarian tumors without LOH at BRCA1 was methylated (Fig. 2, C; Table 1). Thus, more strikingly than in the



**Table 1.** Distribution of BRCA1 promoter hypermethylation in human cancer\*

Primary tumors	Frequency of BRCA1 hypermethylation, No. of tumors/total No. (% of tumors)	<i>P</i>
Breast tumors by histologic types (n = 118)†		
Ductal breast carcinomas	9/73 (12)	
Medullary breast carcinomas	8/12 (67)	.0002‡
Mucinous breast carcinomas	6/11 (55)	.0033‡
Lobular breast carcinomas	0/11	
Papillary breast carcinomas	0/11	
Ductal breast tumors by BRCA1 LOH status (n = 66)		
Breast tumors with LOH at BRCA1	9/45 (20)	.15§
Breast tumors without LOH at BRCA1	1/21 (5)	
Ovarian tumors by BRCA1 LOH status (n = 21)		
Ovarian tumors with LOH at BRCA1	4/13 (21)	.02§
Ovarian tumors without LOH at BRCA1	0/18	
Other tumor types (n = 55)		
Colorectal tumors	0/18	
Liver tumors	0/18	
Leukemias	0/19	

\*LOH = loss of heterozygosity.

†This grouping of breast tumors does not resemble the distribution of histologic types found in the general population because 34 of the nonductal tumors were chosen for study on the basis of their unusual histologic subtypes.

‡Two-tailed Fisher's exact test versus ductal carcinoma.

§Two-tailed Fisher's exact test versus tumors without LOH (separately for breast and ovarian).

breast carcinomas, BRCA1 promoter hypermethylation and loss of the other allele are associated in the ovarian carcinoma ( $P = .02$ ; Fisher's exact test). Together, the data from the breast and ovarian tumors show that the vast majority of tumors with BRCA1 promoter methylation have LOH at this locus, while methylation was rarely observed in tumors without LOH ( $P = .0069$ ; Fisher's exact test, two-tailed).

To test whether or not BRCA1 promoter hypermethylation was limited to only breast and ovarian cancers, the tumor types associated with BRCA1 germline mutations, we also studied neoplasms that are not common in BRCA1 families. None of 18 primary colorectal carcinomas, 18 primary liver carcinomas, or 19 leukemias was methylated at the BRCA1 CpG island (Fig. 2, D; Table 1).

## DISCUSSION

Our study demonstrates that BRCA1 promoter hypermethylation leading to gene silencing is found in nonfamilial, primary breast and ovarian carcinomas. The existence of this epigenetic alteration in BRCA1 supports a role for this tumor suppressor gene in sporadic tumorigenesis in the breast and ovary. The tumor suppressor theory by Knudson et al. (34) predicts that genes that confer a risk of cancer as a result of germline mutations are likely to be somatically mutated in

sporadic cancers of the same type. This prediction has proven to be the case for Rb, p53, VHL, and APC. However, other hereditary cancer genes, such as BRCA2 and ATM, do not appear to conform completely to this model. Also, mutations in the mismatch repair genes hMLH1 and hMSH2 are present only in 10% of the sporadic carcinomas with microsatellite instability (35), the characteristic feature of the tumors developed in patients with hereditary nonpolyposis colorectal carcinoma. More important, for this study, hypermethylation of hMLH1 is a frequent event in sporadic colon and endometrial carcinomas with microsatellite instability (17–19). In a similar way, despite the well-known contribution of germline mutations of BRCA1 in the development of inherited breast and ovarian cancers, somatic BRCA1 mutations are not described in breast tumors and are extremely rare in ovarian carcinoma (6,7).

The two hits referred to in the hypothesis by Knudson et al. that are required for inactivation of tumor suppressor genes are generally thought of as intragenic mutations and loss of chromosomal material (LOH or homozygous deletion). Promoter hypermethylation should now be considered one of the "hits" suffered by tumor suppressor genes (13,14). Thus, silencing by abnormal promoter methylation of Rb, VHL, MLH1, p15, and p16 associated

with inactivation of the other allele by a "classical hit," such as intragenic mutation or LOH, is a relatively common finding in human cancer (13,14). Our findings fit this model, demonstrating the strong association between BRCA1 promoter hypermethylation and the existence of LOH at the BRCA1 locus. These data suggest that, in primary tumors, one allele is lost by deletion and the other is inactivated by aberrant methylation, both events simultaneously leading to the biallelic inactivation and complete lack of function of the BRCA1 gene. This association was clearly seen only when the data from breast and ovarian tumors were considered together. Of interest is the fact that the rate of LOH at the BRCA1 locus is higher than the percentage of tumors methylated at BRCA1 and suggests other avenues of research. Another gene close to BRCA1 may be the primary target of this deletion in some cases. Losing only one allele may also cause a gene-dose effect. Finally, inactivation of BRCA1 may occur by mechanisms other than promoter methylation.

Our data suggest that the role of BRCA1 in noninherited tumors is limited to breast and ovarian tumors, similar to the pattern observed in carriers of germline BRCA1 mutations. Thus, BRCA1 promoter hypermethylation was found only in the breast and ovarian carcinomas and was not observed in other tumor types, including colorectal and liver carcinomas and leukemias. The colorectal carcinomas are particularly interesting, since LOH at the BRCA1 locus is not uncommon in this tumor type (36). The absence of BRCA1 methylation suggests a minor role, if any, for BRCA1 in colorectal tumorigenesis, which is supported by the lack of increased susceptibility to colorectal tumors in BRCA1 families. More striking, BRCA1 epigenetic inactivation in sporadic cases also displays an unusual distribution, again similar to that seen in BRCA1 germline mutation carriers, among histopathologic types of breast carcinoma: We have found that BRCA1 promoter hypermethylation is more frequent in the medullary and mucinous subtypes of breast carcinoma. More than half of the breast carcinomas included in these two particular subtypes displayed BRCA1 aberrant methylation. Both histopathologic subtypes are very uncommon (<5%) in an unselected population of sporadic primary breast carcinomas but are overrepresented in the breast carcinomas that oc-

cur in carriers of BRCA1 germline mutations (31–33). Finally, further linkage between BRCA1 alteration and medullary subtype comes from recent data showing that, while the frequencies of p53 mutations are low among the majority of breast carcinomas, the vast majority of cases of breast carcinomas with inherited BRCA1 mutations and medullary breast carcinomas have alterations in p53 (37–40).

In summary, we have demonstrated that BRCA1 promoter hypermethylation occurs in sporadic breast and ovarian tumors and is associated with LOH and with particular histologic subtypes. Our data now designate a role for BRCA1 as a tumor suppressor gene in the noninherited cases of breast and ovarian carcinomas. This very specific hypermethylation of BRCA1, like VHL in clear-cell renal carcinoma or Rb in retinoblastoma, underscores the central role for this gene in these—and likely only these—tumor types.

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## NOTES

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