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Nonrandom Distribution of Aberrant Promoter Methylation of Cancer-Related Genes in Sporadic Breast Tumors

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ABSTRACT

Purpose: **In an effort to additionally determine the global patterns of CpG island hypermethylation in sporadic breast cancer, we searched for aberrant promoter methylation at 10 gene loci in 54 primary breast cancer and 10 breast benign lesions.**

Experimental Design: **Genomic DNA sodium bisulfate converted from benign and malignant tissues was used as template in methyl-specific PCR for *BRCA1, p16, ESR1, GSTP1, TRβ1, RARβ2, HIC1, APC, CCND2,* and *CDH1* genes.**

Results: **The majority of the breast cancer (85%) showed aberrant methylation in at least 1 of the loci tested with half of them displaying 3 or more methylated genes. The highest frequency of aberrant promoter methylation was found for *HIC1* (48%) followed by *ESR1* (46%), and *CDH1* (39%). Similar methylation frequencies were detected for breast benign lesions with the exception of the *CDH1* gene ($P = 0.02$). The analysis of methylation distribution indicates a statistically significant association between methylation of the *ESR1* promoter, and methylation at *CDH1*, *TRβ1*, *GSTP1*, and *CCND2* loci ($P < 0.03$). Methylated status of the *BRCA1* promoter was inversely correlated with methylation at the *RARβ2* locus ($P < 0.03$).**

Conclusions: **Our results suggest a nonrandom distribution for promoter hypermethylation in sporadic breast cancer, with tumor subsets characterized by aberrant methylation of specific cancer-related genes. These breast cancer subgroups may represent separate biological entities with potential differences in sensitivity to therapy, occurrence of metastasis, and overall prognosis.**

INTRODUCTION

Breast cancer is the most common malignancy in women and represents 18% of all female cancers. The incidence of breast cancer increases with age, and in Western countries the disease is the single most common cause of death among women aged 40–50. The natural history of breast cancer is characterized by a long duration and marked heterogeneity within and between patients. Tumors with similar histopathological appearance can follow significantly different clinical courses and show different responses to therapy. The presence of hormone receptor identifies a subset of patients responsive to endocrine therapy and with better prognosis, but even in this group the clinical outcome can be heterogeneous (1).

Silencing of cancer-associated genes by methylation of CpG islands located at the 5′ end of many genes is thus far the main epigenetic modification identified in tumors. The distribution of the CpG dinucleotide in human genome is generally underrepresented with the exception of genomic regions that usually contain gene promoters or first exons and are almost always unmethylated in adult tissues. Hypermethylation of the CpG islands is associated with delayed replication, condensed chromatin, and inhibition of transcription initiation. A deregulation of DNA methylation is observed in cancer cells that leads to genome-wide hypomethylation and hypermethylation of CpG island associated to cancer-related genes (2).

The global pattern of methylation event in different tumor-types was analyzed with two different approaches. Costello *et al.* (3) used restriction landmarks genomic scanning to determine the methylation profile of CpG islands interspersed in the genome, whereas Esteller *et al.* (4) used a candidate gene approach. In both cases they found that hypermethylation of some of the CpG islands is shared by multiple tumor types, whereas others are methylated in a tumor type-specific manner

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(3, 4). These data suggest that aberrant patterns of methylation are not random and that intra- and intertumor type heterogeneity may display distinguishable methylation subtype.

We have used a candidate gene approach to investigate the methylation profile of primary breast cancer and benign breast lesions. We chose genes that belong to a critical pathway either involved in breast cancer tumorigenesis or methylated in other tumor types. *ESR1*, *RARβ2*, and *TRβ1* belong to the nuclear receptor superfamily and are ligand-mediated transcription factors (5). The protein product of the *APC* gene is an important component of the wingless-type mouse mammary tumor virus integration site signaling pathway, which inactivates β-catenin (6). *CDH1* encodes for a cell surface molecule with a main role in maintaining cell-cell adhesion in epithelial tissues (7). *p16* and *CCND2* are important cell cycle checkpoints (8). *GSTP1* is implicated in the detoxification pathway of xenobiotics and chemotherapeutic agents (9), whereas *BRCA1* is critically involved in cellular response to DNA damage and in the maintenance of genome integrity during DNA synthesis (10). Finally, *HIC1* is a transcription factor with an important role in embryonic development (11). For each of these individual genes it has been reported previously that promoter CpG islands are methylated in cancer cells and expression of the gene is silenced (4). For the *RARβ2* and the *ESR1* genes the silencing could be partially relieved *in vitro* by demethylation of the promoter region after treatment with 5-deoxy-azacytidine (12–14).

Our analysis indicates that aberrant promoter methylation does not occur randomly in breast cancer. Indeed, cancer-related genes are targeted in a specific fashion with a direct correlation among *ESR1*, *CDH1*, *TRβ1*, *GSTP1*, and *CCND2* promoter methylation and an inverse correlation between *RARβ2* and *BRCA1* methylation. Similar patterns were also detected in breast benign lesions, suggesting that promoter hypermethylation of specific genes is an early event in breast cancer development.

MATERIALS AND METHODS

Specimens and DNA Extraction. Fifty-four invasive primary breast cancer tumors (44 ductal and 10 lobular) were collected at the Area of Pathology, University Campus BioMedico (Rome, Italy), at the Clinical Experimental Oncology Laboratory, National Cancer Institute (Bari, Italy), and at the Department of Pathology The Johns Hopkins University, (Baltimore, MD) with Institutional Review Board approval. Thirty-four of the samples were from fresh-frozen specimens, whereas the remaining 20 samples were paraffin embedded. Ten benign breast lesions were obtained as paraffin-embedded slides from the Department of Pathology University "Federico II" (Naples, Italy). The histological type and grade of the tumors were classified according to the World Health Organization criteria. Fresh-frozen samples were carefully dissected on a cryostat so that the tumor samples contained at least 70% of neoplastic cells (15). Paraffin sections were processed as described previously (16).

Methyl-Specific PCR. Genomic DNA sodium bisulfate conversion of the unmethylated cytosine residue to thymidine was performed as described previously (17). The bisulfate converted DNA was used as a template for methyl-specific PCR using primers specific for either the methylated or the modified unmethylated sequences (18). Primer sequences for *ESR1* (19), *p16* (18), *GSTP1* (20), *BRCA1* (21), *RARβ2* (22), *TRβ1* (23), *HIC1* (24), *APC* (6), *CCDN2* (25), and *CDH1* (18) were as described previously. PCR reactions were carried out in a total volume of 40 μl, containing 3 μl of modified DNA, 300 ng of each of the primers, 4.5 μl of PCR buffer (16), 1.25 mM deoxynucleotide triphosphate (Life Technologies, Inc., Rockville, MD), and 0.3 μl PlatinumTaq DNA polymerase (Life Technologies, Inc.; Ref. 19). PCR conditions were as follows: 35 cycles at 95° for 1 min, 54°C-64°C depending on primer pairs for 1 min, and 72°C for 1 min. For each PCR reaction CpGenome Universal Methylated DNA (Serologicals Corp., Norcross, GA) was used as positive control. About half of the reaction volume was run on Tris-borate EDTA 10% Criterion Gels (Bio-Rad Laboratories Inc., Hercules, CA) stained with ethidium bromide and visualized at UV light.

Statistical Analysis. Frequencies with which other loci were methylated when a particular locus was either methylated or unmethylated were compared by the Mann Whitney *U* test as reported previously (26). Comparison of methylation frequencies between two loci was performed using the Fisher exact test. Mean values were compared using the ANOVA. Kaplan-Meier analysis was used to evaluate differences in survival between patients groups. Differences were considered statistically significant for $P < 0.05$.

RESULTS

Aberrant Promoter Methylation in Breast Cancer and Benign Lesions. We searched for aberrant promoter methylation at 10 gene loci (*ESR1*, *BRCA1*, *TRβ1 p16*, *HIC1*, *RARβ2*, *CCND2*, *APC*, *GSTP1*, and *CDH1*) in 54 primary breast cancers and 10 benign lesions (Fig. 1). Aberrant promoter methylation for at least 1 gene was found in 46 of the 54 (85%) breast cancers and in 7 of the 10 (70%) benign lesions.

In malignant tumors, all of the gene loci tested showed methylation in significant proportion of the samples (Table 1). The highest frequencies of aberrant promoter methylation were found for *HIC1*, *ESR1*, *CDH1*, *TRβ1*, *APC*, and *RARβ2* loci, methylated in 48%, 46%, 39%, 28%, 28%, and 20% of the cases, respectively. Lower frequencies were detected for *p16*, *BRCA1*, *GSTP1*, and *CCND2* (18%, 17%, 13%, and 11% respectively; Table 1). The analysis of benign lesions showed minor differences in promoter hypermethylation frequencies for each of the individual genes as compared with malignant lesions (Table 2). The only exception was the *CDH1* gene, for which the difference in frequency between benign (0%) and malignant (39%) tumors was statistically significant ($P < 0.02$).

Analysis of Methylation Distribution in Malignant Tumors. Of the 54 malignant tumors 29 (54%) were methylated at 3 or more loci (mean age ± SE, 56.56 ± 2.82); 11 tumors (20%) displayed 2 methylated loci (mean age ± SE, 60.10 ± 3.31); and 14 tumors (26%) showed 1 or 0 methylated gene (mean age ± SE, 54.21 ± 3.04; Table 1). The analysis of methylation distribution in the 46 breast cancers with at least 1 methylated gene demonstrated a statistically significant association between methylation of the *ESR1* promoter, and methylation at *CDH1* ($P = 0.001$), *TRβ1* ($P = 0.02$), *GSTP1* ($P =$

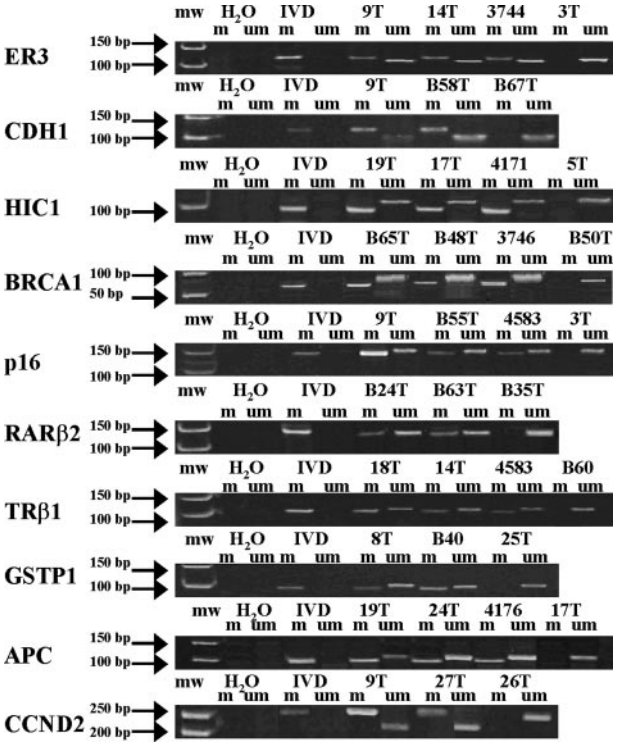

Fig. 1 Representative results of methyl-specific PCR analysis for *ESR1* (ER3), *CDH1*, *HIC1*, *BRCA1*, *p16*, *RARβ2*, *TRβ1*, *GSTP1*, *CCND2*, and *APC*, in sporadic breast cancer and breast benign lesions. Lanes *u* and *m* correspond to unmethylated and methylated DNA, respectively. In each case *in vitro*-methylated DNA (*IVD*) was used as positive control for methylation. *mw*, molecular weight marker; *H_2O*, water control for contamination of the PCR reaction.

0.03), and *CCND2* ($P = 0.007$) loci (Fig. 2). *BRCA1* promoter aberrant methylation was negatively associated with methylation at the *RARβ2* locus. Of the 20 breast cancers with methylation in either *BRCA1* ($n = 9$) or *RARβ2* ($n = 11$), none showed methylation of both loci ($P = 0.03$). No differences in methylation frequency were found between paraffin-embedded and fresh-frozen tissues; this excludes that the differences in methylation distribution might be due to technical artifact.

There was no apparent association between methylation distribution phenotypes and grade, stage, or lymph node status, and no correlations were found for single loci. Kaplan-Meier analysis of patients survival in the 23 cases for which follow-up data were available (mean follow up, 85.87 ± 8.21 months) showed an inverse correlation between high level of methylation and disease-free survival, but it did not reach statistical significance (data not shown).

Correlation between *ESR1* Aberrant Methylation and Protein Expression by Immunohistochemistry. *ESR1* promoter methylation was studied using three primer pairs, ER3, ER5, and ER6, from Lapidus *et al.* (19), covering CpG between position +225 and +529 from the transcription start site. Methylation for all three of the primer pairs was found in 18 of the 25 *ESR1* methylated breast cancers. In 7 cases methylated reactions were positive for 2 ($n = 5$) or 1 ($n = 2$) of the primers. Data from estrogen receptor routine immunohistochemistry were available for 48 of the 54 breast cancers. Of these tumors, 27 were estrogen receptor positive, 5 were weakly positive, and 16 were estrogen receptor negative. *ESR1* CpG island promoter methylation was found in 13 estrogen receptor-positive tumors, 4 weakly positive tumors, and 4 estrogen receptor-negative tumors (21 of the 25 ESR1 methylated tumors). Of the unmethylated tumors, 14 were estrogen receptor positive, 1 weakly expressing the receptor, and 12 were estrogen receptor negative (27 of the 29 ESR1 unmethylated tumors).

DISCUSSION

In an attempt to better understand the epigenetic events that lead to breast cancer development and progression, we have examined the methylation status of multiple loci in primary breast cancer and breast benign lesions. Methylation for at least 1 gene was found in 85% of the breast cancer and in 70% of the breast benign lesions. However, we found significant differences in methylation frequencies among the malignant tumors analyzed. Approximately half of those tumors showed >3 methylated genes, whereas the remaining tumors displayed lower level or no methylation at all (Table 1). These differences did not correlate with age of the patient at the diagnosis suggesting that they are not due to age-related methylation changes but are expression of different levels of deregulation of the mechanisms that protect against CpG island hypermethylation.

A more detailed analysis of our result revealed that CpG promoter hypermethylation does not occur randomly in breast cancer. Nass *et al.* (27) have reported previously coincident methylation of the *CDH1* and *ESR1* genes. Our results confirm this association, but we also identified 3 other genes, *GSTP1*, *CCND2*, and *TRβ1*, with a statistically significant association with *ESR1* promoter hypermethylation. This association was independent from the overall frequency of promoter hypermethylation suggesting that it represents a molecular feature of a subset of breast tumors. The absence of correlation for other genes frequently methylated, such as *HIC1* (*48%*) and *APC* (28%; Table 1), additionally supports this hypothesis.

Another interesting result of our molecular survey is the inverse correlation between *BRCA1* and *RARβ2* aberrant promoter methylation. When we reviewed the literature, this result was consistent with a study by Esteller *et al.* (28) that analyzed 106 sporadic breast tumors at 5 gene loci (*p16*, *CDH1*, *RARβ2*, *GSTP1*, and *BRCA1*). Of these tumors, 68 had at least 1 methylated gene, of which 37 displayed methylation of either *BRCA1* or *RARβ2*. Only 3 of those 37 breast cancers showed a concomitant methylation of both gene loci (28). When results from both studies are considered, ~34% of the tumors show hypermethylation of either *BRCA1* or *RARβ2*, but only 2% of them present concurrent methylation of the two loci. These data additionally support the hypothesis that CpG island aberrant promoter methylation does not occur randomly and suggest the existence of specific selection process targeting key tumor suppressor genes.

In the 10 benign lesions we found substantially the same methylation pattern as in malignant tumors suggesting that inactivation of tumor suppressor genes by methylation represents an early event in breast cancer carcinogenesis. Jeronimo *et al.* (29) reported similar results in a series of breast benign lesions analyzed at 5 gene loci. In our series, the only difference

Table 1 Aberrant promoter methylation distribution in sporadic breast cancer

Cases	ESR1	CDH1	TRβ 1	GSTP1	CCND2	BRCA1	RARβ 2	APC	p16	HIC1
High methylation										
9T	MP	MP	MP	MP	MP	UP	UP	MP	MP	MP
18T	MP	MP	MP	MP	UP	MP	UP	MP	MP	MP
14T	MP	MP	MP	UP	UP	UP	MP	MP	MP	MP
17T	MP	MP	MP	UP	UP	UP	UP	UP	UP	MP
11T	MP	MP	MP	UP	UP	UP	UP	UP	UP	MP
30T	MP	MP	MP	UP	MP	UP	UP	UP	UP	UP
27T	MP	MP	MP	UP	MP	UP	MP	UP	MP	UP
29T	MP	MP	UP	UP	MP	UP	MP	UP	UP	UP
B41T	MP	MP	UP	UP	MP	UP	UP	UP	UP	MP
B40T	MP	MP	UP	MP	UP	UP	UP	UP	UP	UP
B58T	MP	MP	UP	UP	UP	UP	UP	MP	UP	MP
B66T	MP	MP	UP	UP	UP	UP	UP	MP	UP	MP
26T	MP	MP	UP	UP	UP	UP	UP	MP	UP	UP
B42T	MP	MP	UP	UP	UP	MP	UP	UP	UP	UP
B51T	MP	MP	UP	UP	UP	MP	UP	UP	MP	MP
B30T	MP	UP	MP	UP	UP	MP	UP	UP	UP	UP
B45T	MP	UP	MP	UP	UP	UP	UP	UP	UP	MP
B37T	MP	UP	MP	UP	UP	UP	UP	UP	UP	MP
8T	MP	UP	MP	MP	UP	UP	UP	MP	UP	MP
B32T	MP	UP	UP	UP	MP	UP	MP	UP	MP	UP
B35T	MP	UP	UP	UP	UP	UP	MP	UP	UP	MP
B39T	MP	UP	UP	UP	UP	UP	MP	UP	UP	MP
B67T	MP	UP	UP	MP	UP	UP	UP	UP	UP	MP
19T	UP	MP	UP	UP	UP	UP	UP	MP	UP	MP
B55T	UP	UP	MP	UP	UP	MP	UP	MP	MP	UP
22T	UP	UP	UP	MP	UP	UP	UP	UP	MP	MP
B60T	UP	UP	UP	UP	UP	MP	UP	MP	UP	MP
B57T	UP	UP	UP	UP	UP	UP	MP	MP	MP	MP
B24T	UP	UP	UP	UP	UP	UP	MP	MP	UP	MP
Moderate methylation										
B46T	MP	UP	UP	MP	UP	UP	UP	UP	UP	UP
28T	MP	MP	UP	UP	UP	UP	UP	UP	UP	UP
B44T	UP	MP	UP	UP	UP	UP	UP	UP	UP	MP
3T	UP	MP	UP	UP	UP	UP	UP	UP	UP	MP
23T	UP	MP	MP	UP	UP	UP	UP	UP	UP	UP
B38T	UP	UP	MP	UP	UP	UP	UP	UP	UP	MP
B65T	UP	UP	MP	UP	UP	MP	UP	UP	UP	UP
B48T	UP	UP	UP	UP	UP	MP	UP	UP	UP	MP
20T	UP	UP	UP	UP	UP	UP	UP	UP	UP	MP
25T	UP	UP	UP	UP	UP	UP	MP	MP	UP	UP
B64T	UP	UP	UP	UP	UP	UP	MP	MP	UP	UP
Low methylation										
21T	UP	MP	UP	UP	UP	UP	UP	UP	UP	UP
B61T	UP	UP	UP	UP	UP	MP	UP	UP	UP	UP
B59T	UP	UP	UP	UP	UP	UP	MP	UP	UP	UP
33T	UP	UP	UP	UP	UP	UP	UP	MP	UP	UP
B63T	UP	UP	UP	UP	UP	UP	UP	UP	MP	UP
B27T	UP	UP	UP	UP	UP	UP	UP	UP	UP	MP
24T	UP	UP	UP	UP	UP	UP	UP	UP	UP	UP
5T	UP	UP	UP	UP	UP	UP	UP	UP	UP	UP
10T	UP	UP	UP	UP	UP	UP	UP	UP	UP	UP
B43T	UP	UP	UP	UP	UP	UP	UP	UP	UP	UP
B50T	UP	UP	UP	UP	UP	UP	UP	UP	UP	UP
34T	UP	UP	UP	UP	UP	UP	UP	UP	UP	UP
36T	UP	UP	UP	UP	UP	UP	UP	UP	UP	UP
37T	UP	UP	UP	UP	UP	UP	UP	UP	UP	UP
Unmethylated	29	33	39	47	48	45	43	39	44	28
Methylated	25	21	15	7	6	9	11	15	10	26
%*	46%	39%	28%	13%	11%	17%	20%	28%	18%	48%

Abbreviations: MP, methylated promoter; UP, unmethylated promoter.
* Percentage of tumors that carry methylation at the particular gene.

Table 2 Aberrant promoter methylation distribution in benign breast lesions

Cases	ESR1	CDH1	TRβ 1	GSTP1	CCND2	BRCA1	RARβ2	APC	p16	HIC1	Histopathology
4583	MP	UP	MP	UP	UP	UP	UP	UP	MP	MP	FCD
4176	MP	UP	UP	UP	UP	UP	UP	MP	UP	MP	FCD+FAd
4171	MP	UP	UP	UP	UP	UP	UP	UP	UP	MP	FCD+DP+ADHy
3744	MP	UP	UP	UP	UP	UP	UP	UP	UP	UP	FCD
3746	UP	UP	UP	UP	UP	MP	UP	UP	UP	UP	Fad
4750	UP	UP	UP	UP	UP	MP	UP	UP	UP	UP	FCD+FAd
5560	UP	UP	UP	UP	UP	UP	UP	UP	MP	UP	FAd
3731	UP	UP	UP	UP	UP	UP	UP	UP	UP	UP	FCD
3967	UP	UP	UP	UP	UP	UP	UP	UP	UP	UP	FCD
5366	UP	UP	UP	UP	UP	UP	UP	UP	UP	UP	Fad
Unmethylated	6	10	9	10	10	8	10	9	8	7	
Methylated	4	0	0	0	0	2	0	1	2	3	
%*	40%	0%	10%	0%	0%	20%	0%	10%	20%	30%	

Abbreviations: MP, methylated promoter; UP, unmethylated promoter; FCD, fibrocistic disease; FAd, fibroadenoma; DP, ductal papilloma; ADHy, atypical ductal hyperplasia.

* Percentage of tumors that carry methylation at the particular gene.

in methylation frequency between benign and malignant tumors was found for the *CDH1* gene that has been implicated in tumor progression (27). Aberrant promoter methylation was detected in cases of atypical ductal hyperplasia, a condition considered as premalignant, as well as in cases of fibrocystic disease. In this latter group cases with and without promoter hypermethylation were histologically indistinguishable. It would be interesting to test in retrospective studies whether fibrocystic disease patients with and without promoter hypermethylation may differ for the risk of developing malignant tumors.

Expression of the estrogen receptor protein as determined by immunohistochemistry is a predictive marker for response to hormone therapy. However, up to one third of breast carcinomas lack estrogen receptor at time of the diagnosis and a proportion of cancers that are initially estrogen receptor positive lose receptor during progression (30). We found promoter aberrant methylation in only a subset of tumors negative for estrogen receptor by immunostaining. It is known that promoter methylation is not the only mechanisms required for gene silencing. In breast cancer cell lines, together with aberrant methylation histone deacetylation was also detected, suggesting that both mechanisms are required to reach estrogen receptor silencing (12–14). However, there is another issue to address: the genomic organization of the *ESR1* gene is much more complex than expected (31). In the past 10 years 6 other promoters localized upstream to the first identified in 1988 (32) were found. Thus, *ESR1* expression most likely is the result of the interplay between these promoters and their transcriptional regulators. On the other side, we demonstrate *ESR1* hypermethylation in tumors showing ESR1 expression. Routine immunohistochemistry classifies protein expression as a percentage of positively stained tumor cells. In our series, estrogen receptor expression ranged from 40% to 95%, thus it is possible that tumors may contain subclones with some level of *ESR1* promoter methylation. It remains to be determined in larger randomized case-control trials and using quantitative methylation assay whether these subclones might be responsible for the observed reduction of *ERα* expression with consequent acquisition of hormone resistance in breast cancer patients (30).

Our results shed additional light on the significance of epigenetic modification in breast cancer and delineate distinct breast cancer subsets, involving aberrant methylation of specific tumor suppressor genes. Recent studies have found a correlation between CpG islands promoter hypermethylation and clinical parameters such as prognosis and response to therapy (33, 34). Because epigenetic modifications are potentially reversible, the identification of cancer subsets with different patterns of meth-

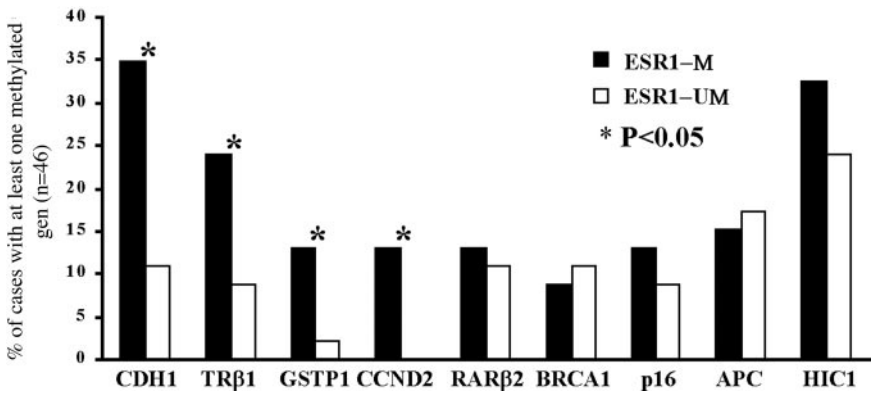

Fig. 2 Correlation between *ESR1* methylated (*ESR1*-M) or unmethylated (*ESR1-UM*) status and hypermethylation at *CDH1*, *TRβ1*, *GSTP1*, *CCND2*, *RARβ2*, *BRCA1*, *p16*, *APC*, and *HIC1* loci.

ylation may result in important consequence for breast cancer patient management.

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