The Journal of Molecular Diagnostics, Vol. 14, No. 6, November 2012

http://dx.doi.org/10.1016/j.jmoldx.2012.07.001

DNA Methylation Index and Methylation Profile of Invasive Ductal Breast Tumors

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Breast carcinogenesis is a multistep process that involves both genetic and epigenetic alterations. Identification of aberrantly methylated genes in breast tumors and their relation to clinical parameters can contribute to improved diagnostic, prognostic, and therapeutic decision making. Our objective in the present study was to identify the methylation status of 34 cancer-involved genes in invasive ductal carcinomas (IDC). Each of the 70 IDC cases analyzed had a unique methylation profile. The highest methylation frequency was detected in the *WT1* (95.7%) and *RASSF1* (71.4%) genes. Hierarchical cluster analysis revealed three clusters with different distribution of the prognostic factors tumor grade, lymph node metastasis, and proliferation rate. Methylation of *TP73* was associated with high histological grade and high proliferation rate; methylation of *RARB* was associated with lymph node metastasis. Concurrent methylation of *TP73* and *RARB* was associated with high histological grade, high proliferation rate, increased tumor size, and lymph node metastasis. Patients with more than six methylated genes had higher rates of relapse events and cancer deaths. In multivariate analysis, *TP73* methylation and the methylation index were associated with disease outcome. Our results indicate that methylation index and methylation of *TP73* and/or *RARB* are related to unfavorable prognostic factors in patients with IDC. These epigenetic markers should be validated in further studies to improve breast cancer management. *(J Mol Diagn 2012, 14:613–622; http://dx.doi.org/10.1016/j.jmoldx.2012.07.001)*

Breast cancer is a heterogeneous disease with varied histopathology, clinical behavior, prognosis, and response to treatment.[1,2] Invasive ductal carcinoma (IDC) comprises approximately 75% to 85% of primary breast cancers and exhibits a distinct biological behavior, compared with invasive lobular carcinoma (ILC), the second most common overall breast cancer type.[3]

In the assessment of breast cancer, the more informative prognostic factors are lymph node status, primary tumor size, and histological grade.[4] In Western countries, the estimated 5-year disease-free survival (DFS) in patients with unaffected lymph nodes is approximately 80%.[5,6] In Argentina, patients with affected lymph nodes experience five times reduced overall survival (OS) rates and almost three times higher recurrence risk.[7] If lymph nodes do not have metastasis, the most important prognostic factor is tumor size, because disease recurrence rate increases as primary tumor size increases.[8] The histological characteristics of tumors can be evaluated, graded, and related to prognosis of the disease. The Nottingham combined histological grade takes into account three characteristics of the tumor: i) differentiation (tubule formation), ii) nuclear pleomorphism (nucleus/cytoplasm relation), and iii) mitotic count (mitoses per high power field). This combined evaluation leads to three grades: low, intermediate, and high.[9,10] Several studies have associated increased histological grade with poor prognosis.[4,11]

During the last decade, the classification of patients with breast cancer by the traditional characteristics has been modified to include use of gene expression signatures, classifying breast tumors into six groups: normal breast-like, luminal A, luminal B, HER2-enriched, claudin-low, and basal-like.[12,13] Nonetheless, strong differences in clinical evolution and treatment response can still be found within these molecular subtypes, which suggests

Supported by the National University of Cuyo with grants for the development of clinical and basic research, School of Medical Sciences (2009 and 06/J343 2009).

Accepted for publication July 6, 2012.

CME Disclosure: The authors of this article and the planning committee members and staff have no relevant financial relationships to disclose.

Supplemental material for this article can be found at *http://jmd.amjpathol.org* or at *http://dx.doi.org/10.1016/j.jmoldx.2012.07.001*.

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that unknown subgroups exist among these classifications. Based on the analysis of pathway signatures, Gatza et al[14] suggested that as many as 18 molecular subtype could exist in breast cancer.

Breast carcinogenesis is considered a multistep process, involving a combination of genetic and epigenetic alterations.[15,16] The most studied epigenetic alteration in human neoplasms is the hypermethylation of CpG islands in gene promoter regions. This is a common mechanism in suppression of cancer-related genes.[17] Strong evidence suggests a relationship between aberrant DNA methylation pattern and clinicopathological features of breast tumors.[18,19] Shinozaki et al[20] showed that glutathione S-transferase pi 1 (*GSTP1*) gene methylation is strongly associated with increased tumor size and sentinel lymph node metastasis. They also showed that methylation of the retinoic acid receptor, beta, gene (*RARB*) was associated with macroscopic sentinel lymph node metastasis, whereas the Ras association (RalGDS/AF-6) domain family member 1 gene (*RASSF1*) was the most frequently methylated in primary breast tumors. Furthermore, Van der Auwera et al[21] have suggested that a CpG island methylator phenotype exists in invasive breast cancer, and they inferred a relation between methylation of genomic regions and prognosis of patients with breast cancer. Identification of aberrantly methylated genes in breast tumors and their relation to clinical parameters thus contributes to a better understanding of the disease and to improving diagnostic, prognostic, and therapeutic decisions for breast cancer patients.

The objective of the present study was to identify alterations in DNA methylation related to invasive ductal carcinomas by evaluating the methylation status of 49 CpG islands localized within 34 cancer-involved genes. Our key findings reveal epigenetic alterations that could contribute to a better understanding of the heterogeneity of breast tumorigenesis, elucidating also the relationship of such alterations with previously known prognostic factors.

Materials and Methods

Patients

From March 2007 to December 2009, a total of 98 breast cancer patients who did not receive neoadjuvant treatment were consecutively enrolled. These patients exhibited pure IDCs, pure ILCs, and mixed tumor type growth patterns (Table 1). Clinical features of patients bearing IDCs are given in Table 2. Six normal breast tissues and three fibroadenomas were included as control samples.[22] Ethical approval was obtained from the Ethics Committee of the School of Medical Sciences, National University of Cuyo, Mendoza, Argentina. All patients signed an informed consent based on the scientific and ethical principles of the World Medical Association's Declaration of Helsinki. Tumors were staged according to the sixth edition of the American Joint Committee on Cancer (AJCC) guide.[23] Histological type, lymph node status, tumor size, and histological grade were assessed by the same pathologist (O.M.T.), and all patients were under the care of the same surgeon (F.E.G.) at the Instituto Gineco-Mamario Medical Center (Mendoza, Argentina). The status of estrogen receptor (ER), progesterone receptor (PR), epidermal growth factor receptor 2 (HER2), and Ki-67 were established by immunohistochemical staining in a single standardized laboratory. In tumors with a score +2 HER2 expression, fluorescence *in situ* hybridization (FISH) was performed to evaluate the copy number alterations in the HER2 encoding gene, *ERBB2*.

Table 1. Pathological Classification of the Analyzed Tumors

Tumor type	No.	%
Total patients	98	100
Invasive ductal carcinoma	70	71.42
Invasive lobular carcinoma	16	16.32
Mixed tumor type*	12	12.24

*Mixed ductal and lobular cells.

Table 2. Main Clinical Characteristics of Patients Bearing Pure IDCs

Characteristics	No.	%
Total patients	70	100
Age (years)*		
>50	36	51.42
≤50	34	48.57
Disease stage		
I	27	38.6
IIA	23	32.9
IIB	9	12.9
IIIA	10	14.3
IIIC	1	1.4
Lymph node metastasis		
Negative	39	55.7
Positive	31	44.3
Histological grade		
1 (low)	12	17.1
2 (intermediate)	30	42.9
3 (high)	28	40
Proliferation rate		
Low (PCNA < 35%)	18	25.7
High (PCNA ≥ 35%)	52	74.3

*Mean age: 54.2 years (SEM = 1.57).

Tissue Dissection and DNA Isolation

After surgery, each sample was divided into two portions: one was for routine pathological analysis using H&E staining, and the other was dissected under a pathologist's (O.M.T.) guidance to enrich tumor cell content and cryopreserved at −80°C. Normal specimens were obtained from surgical margins of benign breast disease tissue (fibroadenoma; $n = 3$) and normal breast tissue ($n = 3$). After at least 24 hours of congelation, DNA was extracted using a cetyl trimethylammonium bromide (CTAB)/chloroform:isoamylic alcohol procedure. Briefly, each sample was broken with a frozen mortar and the homogenate was washed with 3 mL of $T_{10}E_{10}$ buffer (Tris/HCl 10 mmol/L and EDTA 10 mmol/L), pH = 7. The pellet was dissolved in 3 mL CTAB solution [2 g/L CTAB (Sigma-Aldrich, St. Louis, MO), 100 mmol/L Tris/HCl, 0.2% 2-mercaptoethanol, and 20 mmol/L EDTA] and in-

cubated at 60°C for 4 hours. After pellet dissolution, 3 mL of chloroform:isoamylic alcohol (24:1) solution was added and mixed, and then centrifuged at 1200 × *g* for 5 minutes; the aqueous phase was collected and mixed with 6 mL of 100% ethanol. The precipitated DNA was dissolved in $T_{10}E$ buffer (Tris/HCl 10 mmol/L and EDTA 1 mmol/L), pH 8, and stored at −20°C.[22]

MS-MLPA

The methylation status of 34 genes was assessed by 49 different probes, in some cases including more than one CpG island per gene (see Supplemental Table S1 at *http://jmd.amjpathol.org*). For this purpose, methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA) kits were used (ME001B and ME002; MRC-Holland, Amsterdam, The Netherlands). These 34 genes have been found aberrantly methylated in different human tumors, and are unmethylated in healthy cells. In addition, several reference probes were included that do not include CpG sites. The MS-MLPA assays were performed according to the manufacturer's recommendations and as described by Nygren et al,[24] with subtle modifications (ie, separated ligation and restriction steps and extended restriction enzyme incubation time) to avoid background signals.[22] This methodology has been validated previously.[25] Results were analyzed using GeneMarker version 1.75 software (SoftGenetics, State College, PA). The GeneMarker software normalizes the data by dividing the peak area of a single probe by the peak areas of all reference probes; the normalized peaks from the analyzed samples are then compared with the normalized peaks from the control reaction.

Given that the tumor cell content varies among samples, we considered only a binary approach for the methylation status (0 = unmethylated and 1 = methylated). Based on our previous experiments using cell lines, we established a cutoff threshold, considering a region to show methylation if the methylation dosage ratio was ≥8%.[22]

Statistical Analysis

Both χ^2 test and odds ratio analyses were applied to study the associations between CpG island methylation status and clinical variables. The strength of associations was assessed by the ϕ coefficient for dichotomous variables and Cramer's *V* coefficient for polytomous variables.[26] Because of the nonparametric nature of the data, correlations were assessed by Spearman's ρ coefficient. Based on the methylation index (MI), we established an empirically based cutoff value by which the patients were divided into two groups: one group included patients with fewer than six methylated genes per tumor and the other group included patients with six or more methylated genes per tumor.

The Cox proportional hazards regression model was applied to estimate the correlation between aberrant DNA methylation and patient survival. Survival times were calculated from the time of surgery until the event of interest, including DFS and OS. In the initial model, lymph node status, histological grade, tumor size, disease stage, patient age, proliferation rate, and ER, PR, and HER2 expression were entered as covariates. Kaplan-Meier graphing was performed to visualize the disease outcome of the patients. The log-rank test was applied to evaluate significance.

The *P* values were adjusted using Benjamini and Hochberg's approach and a false discovery rate (FDR) for multiple testing; the corrected *P* value was designated as the *q* value. After correction, α values of <0.05 were considered statistically significant. All statistical analyses were performed using SPSS version 17 software (IBM SPSS, Chicago, IL).

We performed unsupervised hierarchical clustering analysis to identify tumors with correlated methylation profiles and genes with correlated methylation patterns across tumors. To decrease the influence of the excess number of data points with a value of zero (no methylation) in our data, we used a taxicab geometry analysis or Manhattan distance (M-dist) and average linkage.[27] Bootstrap resampling using 100 iterations was applied to validate the hierarchical tree samples and gene organization; a bootstrap value of ≥65% was used as the cutoff point for hierarchical cluster analysis. To establish the statistical distance between the methylation profiles, we applied the gene distance matrix (GDM) algorithm with Manhattan distance. To identify genes with methylation associated with tumor characteristics, we used significance analysis for microarray function (SAM) with 4000 permutations. To keep the median number of false significant genes at zero, we changed the delta value (δ) in each case and we informed in the text. Hierarchical clustering, bootstrap tree support, and GDM and the SAM analyses were performed using MultiExperiment Viewer MeV version 4.7 software (TM4 Group; Dana Farber Cancer Institute, Boston, MA).[28]

Results

The Epigenetic Signature Is Specific for Each Invasive Ductal Carcinoma

The MS-MLPA analysis of control samples revealed that all 34 genes were unmethylated in normal breast tissue ($n = 6$) and also in fibroadenoma tissue ($n = 3$). Remarkably, however, all 98 of the breast tumors exhibited aberrant methylation in at least 2 of the 34 genes. To analyze a tumor population with similar clinical characteristics, we selected only pure invasive ductal carcinomas (IDCs) ($n = 70$). We observed that each tumor exhibited a unique aberrant DNA methylation profile. This observation was confirmed by using the gene distance matrix (GDM) algorithm. All of the IDCs exhibited a unique epigenetic signature (Figure 1A). The highest similarity (minimal statistical distance) was observed between the profiles of IDC 43 and IDC 48 (M-dist = 0.04), which shared the methylation status of 48/49 CpG islands (Figure 1B).

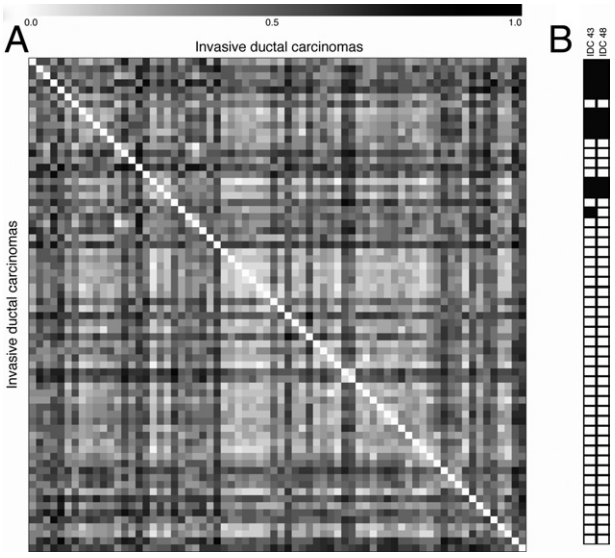

Figure 1. Statistical distances between epigenetic signatures of IDCs. **A:** Sample distance matrix generated using MultiExperiment Viewer MeV software. The two-color scale indicates the scaled Manhattan distance between the epigenetic signatures of 70 IDCs. Absolute identity (M-dist = 0) is shown white; the greatest distance (M-dist = 1) is shown in black; intermediate distances are shown in shades of gray. **B:** Epigenetic signatures of two IDCs (cases 43 and 48) with the minimal Manhattan distance. Black squares indicate methylated regions; white squares indicate unmethylated regions.

WT1 *and* RASSF1 *Are Frequently Methylated in IDC*

We observed that methylation of some CpG islands appeared more often involved in invasive ductal carcinogenesis. A CpG island located within the promoter region of the Wilms tumor 1 gene (*WT1*, −411 bp before ATG bp) was methylated in 67 of 70 IDCs (95.7%). The *RASSF1* gene was analyzed in two different CpG islands (−136 bp and + 46 bp), of which one was methylated in 50/70 IDCs (71.4%) and the other in 46/70 IDCs (65.7%). We also observed a group of CpG islands that were rarely or never methylated in IDCs, notably involving *VHL* (+115 bp) (1/70, 1.4%) and *MLH1* (+320 bp) (0/70, 0%) (Figure 2).

The methylation status of some of the most frequently methylated genes was confirmed using alternative techniques. For *TP73* (alias *p73*), we used methylation-specific PCR (see Supplemental Figure S1 at *http://jmd.amjpathol.org*); for *RASSF1* we used nested methylation-specific PCR, as described previously.[22]

Invasive Ductal Carcinomas Are Organized in Hierarchical Clusters Based on the Epigenetic Information

The methylation profile of the 70 IDCs was subjected to unsupervised hierarchical clustering with Manhattan distance and average linkage. For this analysis, to decrease the influence of unmethylated events and the consequent excess of zeros in the data, we selected 17 genes that were methylated in more than 15% of the IDCs.

Cluster analysis produced three clusters distinguishing the patients (Figure 3). Cluster 1 was characterized by methylation of *PAX5*, *PAX6*, *RASSF1*, *APC*, and *GSTP1* and the unmethylation of the remaining genes. Cluster 3 was characterized by methylation of *CD44*, *CADM1*, *TP73*, *ESR1*, and *TP15* and the unmethylation of *PAX5*, *PAX6*, *RASSF1*, *APC*, and *GSTP1*. Finally, cluster 2 included IDCs with methylation of both groups of genes

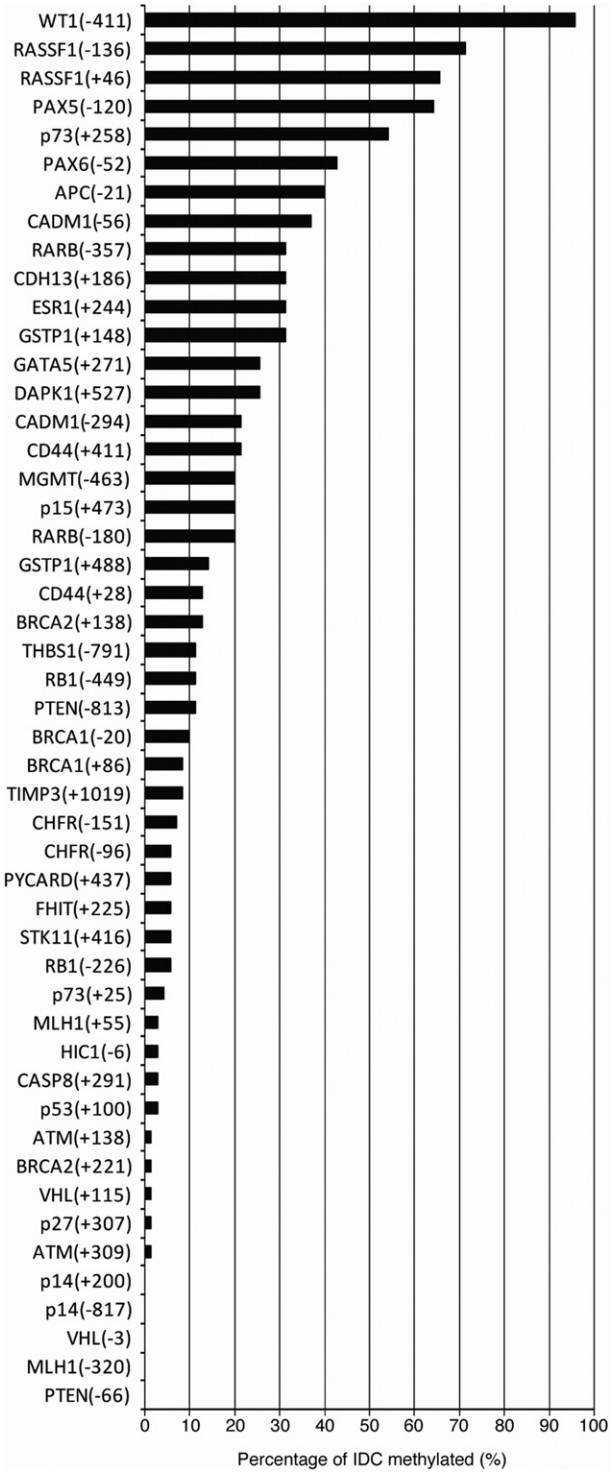

Figure 2. Frequency distribution of the presence of methylation (defined as dosage ratio ≥8%) for each of 49 analyzed CpG islands among 70 IDCs.

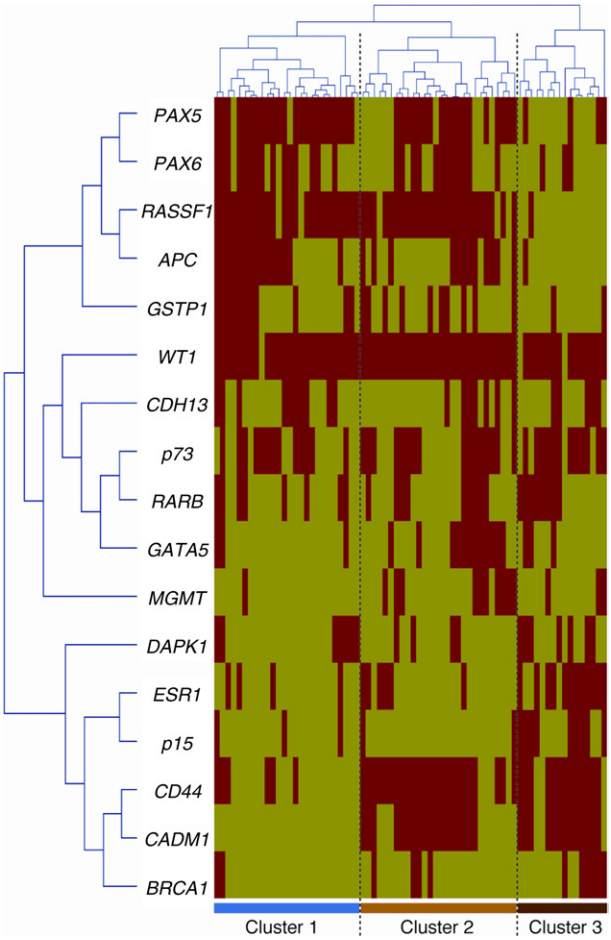

Figure 3. Unsupervised hierarchical cluster analysis of the epigenetic signature of 70 IDCs using the information from 17 genes. The heat map was generated using MultiExperiment Viewer MeV software. A bootstrap value of ≥65% was considered to define a cluster. Red indicates methylated status, green indicates unmethylated status.

and the unmethylation of *TP15*. *WT1* was methylated throughout all three clusters.

The distribution of prognostic factors in these clusters was as follows: high tumor grade was present in 62% of patients in cluster 3, 27% in cluster 1, and 39% in cluster 2; lymph node metastasis was present in 62% of patients in cluster 3, 46% in cluster 1, and 32% in cluster 2; and high proliferation rate was present in 87% of patients in cluster 3, 69% in cluster 1, and 71% in cluster 2. Despite the observed trends, after statistical analysis and FDR corrections these differences were not statistically significant, probably because of the reduced number in samples in each group.

The Methylation Status of TP73 *and* RARB *Is Associated with Clinical and Pathological Factors*

To identify genes with methylation status significantly associated with clinical prognostic factors, we used significance analysis of microarrays (SAM) algorithms. Analysis of the distribution of methylation status of genes across the different histological grades showed that the only gene in which methylation was related to histological grade was *TP73* (SAM $\delta = 0.26$). Association analysis using χ^2 test and Cramer's *V* coefficient confirmed the association of *TP73* methylation and high histological grade: *TP73* methylation was present in 82.1% of grade 3, 46.7% of grade 2, and only 8.3% of grade 1 tumors (Figure 4, A and D; Table 3).

Analysis of the distribution of methylated genes between IDCs with high versus low proliferation rate suggests that methylation of *TP73* and *CDH13* genes was associated with high proliferation rate in tumors (SAM $\delta = 0.77$). Association analysis revealed that IDCs with high proliferation rates exhibited a significantly higher frequency of *TP73* methylation: 67.3% for high proliferation rate tumors versus 16.7% for low proliferation rate tumors (odds ratio = 8.28, 95% CI = 2.45 to 29.93) (Figure 4, B and D; Table 3). Methylation of the *CDH13* gene exhibited a trend toward association with high proliferation rate tumors ($\phi = 0.306$, $P = 0.011$, $q = 0.12$).

The distribution of methylated genes between patients with affected and unaffected lymph nodes revealed that methylation of the *RARB* gene was associated with patients with lymph node metastasis (SAM $\delta = 0.6$). *RARB* (−357 bp) methylation was observed in 54.8% of patients with affected lymph nodes, compared with only 10.3% of patients with unaffected lymph nodes (odds ratio = 5.32, 95% CI = 1.73 to 16.36) (Figure 4, C and E; Table 3).

Histological grading (grades 1, 2, and 3) was not associated with methylation of *RARB* (−357 bp). However, analysis of methylation of *RARB* (−357 bp) in grade 3 versus grade 1 tumors revealed a trend toward association: 46.4% in grade 3 versus 0% in grade 1 tumors ($\phi = 0.454$, $P = 0.004$, $q = 0.054$); however, the trend lost significance after FDR multiple comparison correction.

The Concurrent Methylation of TP73 *and* RARB *Is Associated with Poor Prognostic Factors*

Unsupervised hierarchical cluster analysis of the genes revealed three main branches, supported by bootstrap values of ≥65%, (Figure 3; see also Supplemental Figure S2 at *http://jmd.amjpathol.org*). In cluster 1, we observed four genes (*PAX5*, *PAX6*, *RASSF1*, and *APC*); in cluster 2, three genes (*TP73*, *RARB*, and *GATA5*); and in cluster 3, five genes (*ESR1*, *TP15*, *CD44*, *CADM1*, and *BRCA1*).

The distance between the gene branches indicates the level of association between two genes. The genes with the closest association were *CD44-CADM1* (M-dist = 0.21), followed by *PAX5-PAX6* (M-dist = 0.29), *TP15-ESR1* (M-dist = 0.32), *RASSF1-APC* (M-dist = 0.33), and *TP73-RARB* (M-dist = 0.33).

Because *TP73* and *RARB* belong to the same cluster, with a short statistical distance, and because both were independently associated with poor prognostic factors of breast cancer, we hypothesized that patients whose tumors have both genes methylated will have a poorer prognostic profile than patients whose tumors have both genes unmethylated. Concurrent methylation showed a significant and strong association with high histological grade, high

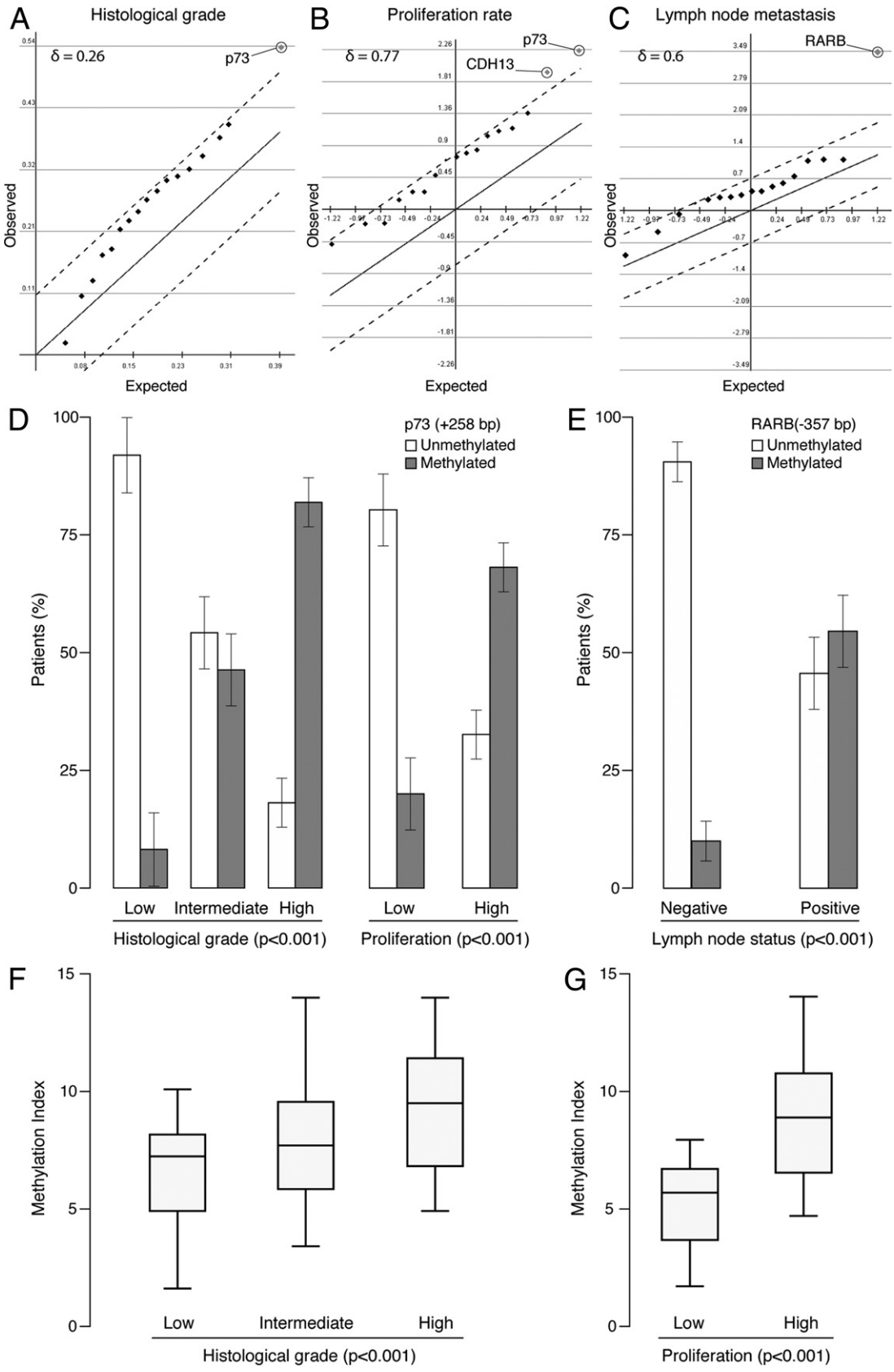

Figure 4. Relations between epigenetic alterations and prognostic factors. SAM analysis for methylation of each gene and histological grade (**A**), proliferation rate (**B**), and lymph node metastasis (**C**). Percentage of patients with methylation of *TP73* versus histological grade and proliferation rate (**D**), and with methylation of *RARB* versus lymph node status (**E**). Distribution of the number of methylated genes within histological grade (**F**) and proliferation rate (**G**). Data are expressed as mean proportion ± SEM (**D–E**) or as minimum, lower quartile, median, upper quartile, and maximum (**F** and **G**).

Table 3. Statistical Relationships between Breast Cancer Prognostic Factors and Qualitative and Quantitative Epigenetic Characteristics

Prognostic factor	*TP73* methylation	*RARB* methylation	Concurrent *TP73* and *RARB* methylation	Methylation index
Histological grade	Cramer's $V = 0.488$; $P < 0.001$ ($q = 0.007$)	—	Cramer's $V = 0.657$; $P < 0.001$ ($q = 0.003$)	Spearman's $\rho = 0.400$; $P < 0.001$ ($q = 0.009$)
Proliferation rate (low versus high)*	$\phi = 0.444$; $P < 0.001$ ($q = 0.008$)	—	$\phi = 0.454$; $P = 0.002$ ($q = 0.024$)	Spearman's $\rho = 0.459$; $P < 0.001$ ($q = 0.008$)
Tumor size, in cm	—	Spearman's $\rho = 0.246$; $P = 0.040$ ($q = 0.37$)†	Spearman's $\rho = 0.335$; $P = 0.025$ ($q = 0.28$)†	$P > 0.05$
Lymph node metastasis; (negative versus positive)	—	$\phi = 0.450$; $P < 0.001$ ($q = 0.008$)	$\phi = 0.474$; $P = 0.001$ ($q = 0.012$)	Spearman's $\rho = 0.257$; $P = 0.032$ ($q = 0.32$)†
Methylation index	Spearman's $\rho = 0.474$; $P < 0.001$ ($q = 0.007$)	Spearman's $\rho = 0.477$; $P < 0.001$ ($q = 0.007$)	Spearman's $\rho = 0.688$; $P < 0.001$ ($q = 0.002$)	Spearman's $\rho = 1.000$

*Low = PCNA <35%; high = PCNA ≥35%.
†Not significant.

proliferation rate, increased tumor size, and lymph node metastasis (Table 3). Another characteristic of tumors with concurrent methylation of *TP73* and *RARB* was a strong association with a high number of methylated genes (higher MI) (Table 3).

Tumors with Poor Prognostic Factors Exhibit a High Methylation Index

The MI was calculated as the sum of methylated genes per sample. The minimum MI was 2 and the maximum was 14 (mean, 7.73; SEM = 0.37). We assessed the correlation between MI and different clinical prognostic factors. Spearman's ρ coefficient revealed that tumors with high MI exhibited high histological grade and high proliferation rate (Figure 4, F and G; Table 3).

Patient Outcome Analysis

The median follow-up duration for this patient cohort was 44.6 months (SD = 10.2; range, 2.6 to 69.6); the most frequent disease stage was stage I (38.6%) (Table 2). During this period, 66 patients were monitored, of whom 15 experienced any relapse event and 8 died (see Supplemental Figure S3 at *http://jmd.amjpathol.org*). For the outcome analysis, we divided the patients into two groups based on the methylation index (MI < 6 versus MI ≥ 6). We observed a higher frequency of relapse events and cancer mortality in the second group (Table 4). Analysis of DFS and OS showed a trend toward association between tumors with six or more genes and worse DFS (log rank $\chi^2 = 3.84$, $P = 0.05$, $q = 0.37$) but not with OS (log rank $\chi^2 = 0.518$, $P = 0.472$) (Figure 5).

Patients whose tumors exhibited methylation of either *TP73* or *RARB* had higher frequency of relapse events and cancer mortality than patients with both genes unmethylated (Table 4). Analysis of DFS and OS and the methylation status of *TP73* and *RARB* did not reveal statistical differences (see Supplemental Figure S4 at *http://jmd.amjpathol.org*).

Multivariate Cox proportional hazards analysis suggested that the following factors were independently associated with DFS: MI, histological grade, disease stage, and ER expression (Table 5). *TP73* methylation and HER2 expression were not independently significantly associated with DFS, but are included in the most significant model. The final model for OS indicated that only disease stage was independently predictive of survival; however, the final model included the methylation status of *TP73* (Table 5).

Discussion

Characterization of epigenetic aberrations in primary breast tumors can be an important marker related to disease pathogenesis. Previous studies have demonstrated that methylation of different genomic regions is related to good or poor prognosis of breast cancer. Methylation of the *PTEN* gene is related to high histological grade, high HER2 (*ERBB2*) expression, and increased tumor size in breast cancer.[29] Others have found that large tumor size, advanced tumor stage, and lymph node metastasis are related to *TP16* gene methylation.[30] Furthermore, some studies have found a correlation between poorly differentiated tumors and a high number of methylated CpG islands.[31,32] Taken together, these observa-

Table 4. Outcome According to Epigenetic Alterations in Patients Bearing Pure IDCs

	Methylation index		*RARB* and *TP73* methylation	
	<6	≥6	Both unmethylated	One or two methylated
Patients with follow-up [no. (%)]	23 (34.8)	43 (65.1)	29 (43.9)	37 (56.1)
Median follow-up (months ± SD)	42.6 ± 8.5	36.7 ± 12.8	43.6 (7.1)	36.9 (13.9)
Relapse events [no. (%)]	2 (8.7)	13 (30.2)	5 (17.2)	10 (27)
Deaths [no. (%)]	2 (8.7)	8 (18.6)	2 (6.9)	7 (18.9)

No follow-up data were available for 4 of the 70 patients.

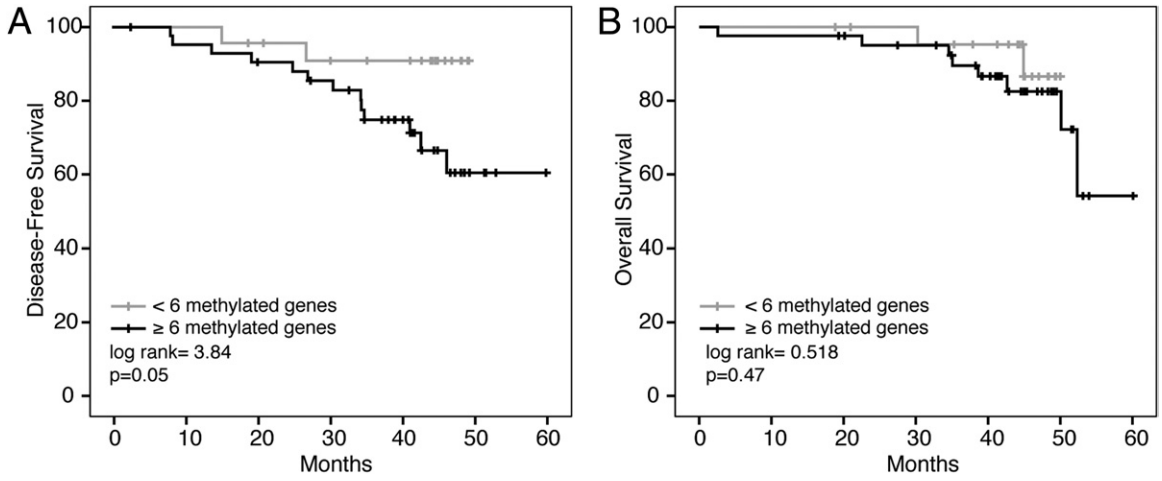

Figure 5. Kaplan-Meier curves show the distribution of DFS grouped by patients with MI ≤ 6 versus MI > 6 (**A**) and OS grouped by patients with MI ≤ 6 versus MI > 6 (**B**).

tions contribute to a better understanding of the etiology of tumor progression.

In the present study, our objective was to analyze the methylation status of 34 cancer-involved genes and to compare this information with clinical prognostic factors. We applied the MS-MLPA assay to test the methylation status of 49 CpG islands located within 34 cancer-related genes.

Epigenetic alterations occur not in a single gene, but in a network of genes, and studies based on simultaneous analysis of many genes contribute wider knowledge. Several reports have shown that the DNA methylation profile is specific for each type of human tissue.[33–35] Our analysis revealed epigenetic differences among invasive breast tumors that share a common histological type (ductal). From our analysis of 34 genes in 70 IDC tumors, we discovered that each IDC exhibits a unique methylation profile. Despite the specificity of the tumor methylation profile, many of these patients are classified into the same subtype and in consequence they receive the same treatment. These epigenetic differences among IDCs could be reflected in the heterogeneous treatment response observed among breast cancer patients and might also reflect the variety of environmental influences during tumor progression (eg, hormonal, reproductive history, genetic background, and carcinogen exposure).[36–39]

Even though the individual methylation signatures are unique, our results show that IDCs overall share frequent methylation of certain regions, including *WT1*, *RASSF1*, *APC*, *PAX5*, and *PAX6*. The methylation frequencies of *RASSF1* (71.4%), *RARB* (31.4%), and *DAPK1* (27.1%) in the present study are similar to the frequencies reported by Van der Auwera et al,[40] who found that *RASSF1* was methylated in 74% of breast tumors, *RARB* in 29%, and *DAPK1* in 31%. To our knowledge, the present study is the first to identify a high frequency (95.7%) of *WT1* methylation in breast cancer. On the other hand, we observed that some regions are rarely involved (eg, *ATM*, *MLH1*, *VHL*, and *TP14*). Future validation studies could be developed using a designed MLPA-based assay that includes only the specific regions for analysis of IDC pathogenesis, similar to an approach developed for bladder cancer.[41] This would allow a low-cost and rapid screening of breast cancer patients for key significant methylation aberrations in genes.

Unsupervised hierarchical clustering analysis revealed three main clusters among the patients, who differed in tumor grade, proliferation rate, and lymph node metastasis. Cluster 3 was characterized by higher frequencies of poor prognostic factors, compared with clusters 1 and 2.

Histological grading of breast tumors is of significant importance for treatment choices. We detected that increased MI and methylated *TP73* were independently associated with high-grade tumors. We observed a trend toward association between *RARB* methylation and high-grade tumors, although it was not significant after multiple comparisons correction. A substantial proportion (30% to 60%) of breast tumors are classified as histological grade 2,[42] which is not very informative for treatment decision making. In these cases, therefore, the choice of treatment is generally influenced by other prognostic fac-

Table 5. Cox Proportional Hazard Analysis of Epigenetic Alterations

Parameter	*P* value	HR	95% CI
Disease-free survival (χ^2 = 18.901, df = 6, *P* = 0.004)			
Methylation index, high	0.016	1.26	1.05–1.53
Histological grade, high	0.024	3.33	1.17–9.44
ER expression, positive	0.041	0.28	0.09–0.95
Disease stage, advance	0.046	1.53	1.01–2.33
TP73, methylated	0.116	6.74	0.62–72.72
HER2 expression, positive	0.123	5.81	0.61–55.55
Overall survival (χ^2 = 11.039, df = 2, *P* = 0.004)			
Disease stage, advanced	0.007	2.14	1.23–3.69
TP73, methylated	0.151	3.24	0.65–16.16

CI, confidence interval; df, degrees of freedom; ER, estrogen receptor; HER2, epidermal growth factor receptor 2 (*ERBB2* gene); HR, hazard ratio.

tors. When grade 2 tumors were excluded from the statistical analysis, we detected increased strength of the associations between histological grade and the number of methylated genes, as well as between histological grade and methylated *TP73* (data not shown). Detection of epigenetic markers that distinguish between low and high histological grade could be useful for clarifying such intermediate cases and thereby contribute to treatment selection.

Our results suggest that tumors with high and low proliferation rates are epigenetically different entities. Methylation of *TP73* and a high MI are two epigenetic alterations that were strongly associated with high proliferation rates. *CDH13* methylation also exhibited a trend toward association with highly proliferating tumors.

The present study showed an association between the unmethylation of the *RARB* gene and absence of lymph node metastasis. This finding is in accord with previous observations of *RARB* methylation in tumors from patients with lymph node metastasis.[20,43] *RARB* encodes the retinoic acid receptor β (RAR-β), a member of the thyroid-steroid hormone receptor superfamily of nuclear transcriptional regulators. This is a nuclear receptor that binds retinoic acid, the biologically active form of vitamin A, which mediates cellular signaling in embryonic morphogenesis, cell growth, and differentiation. The lack of *RARB* expression is related to an enhanced mitotic activity and loss of differentiation. It is thought that the RAR-β protein limits growth of multiple cell types by regulating gene expression. Our data suggest that aberrant methylation of *RARB* correlates with poor prognostic factors, evidence in support of the tumor suppressor role of *RARB* in the progression of ductal tumorigenesis. Patients bearing tumors with *RARB* promoter methylation may benefit from targeted chemopreventive treatment with a combination of all-*trans*-retinoic acid (ATRA) and demethylating agents (eg, procaine),[44] as well as histone deacetylase inhibitors (eg, trichostatin A)[45] that could reactivate the *RARB* gene expression.

Patients with poor prognostic factors exhibit both quantitative (MI) and qualitative (*TP73* and/or *RARB*) epigenetic alterations. The novel finding of concurrent methylation of *TP73* and *RARB* reveals that these alterations are not at random. *TP73* is involved in cell cycle regulation and induction of apoptosis. Debate persists about the exact function of *TP73*. Recent studies revealed that p73 protein is present in early stages of neurological development and counters neuronal apoptosis by blocking the proapoptotic function of p53.[46] This strongly suggests that, like *RARB*, *TP73* also plays a large role in cellular differentiation. We suggest that IDCs with concurrent methylation of *TP73* and *RARB* are more susceptible to acquiring a highly proliferative and dedifferentiated phenotype that leads to a more aggressive disease.

We observed more relapse events in patients with a high number of methylated genes and with concurrent methylation of *TP73* and *RARB* in their primary tumors, compared with patients without these epigenetic alterations. The final multivariate model for DFS includes MI and *TP73* methylation status; for OS, it includes *TP73* methylation.

In attempting to analyze only fresh tumor samples, all of the patients were included in a prospective approach. This prospective nature of the study is the main limitation to any conclusion of whether the MI and/or methylation of *TP73-RARB* are factors for predicting prognosis, because of the limited duration of follow-up. The median tumor stage was I, and the median follow-up was 44.6 months, which is short for follow-up of early-stage breast cancer disease.

The present findings indicate that MI and methylation of *TP73* and/or *RARB* are related to unfavorable prognostic factors of patients with IDCs. These epigenetics markers could therefore be included in further studies to improve the molecular diagnosis of patients with IDC.

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