High Promoter Methylation Levels of *APC* Predict Poor Prognosis in Sextant Biopsies from Prostate Cancer Patients

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Abstract

Purpose: Prostate cancer is a highly prevalent malignancy and constitutes a major cause of cancer-related morbidity and mortality. Owing to the limitations of current clinical, serologic, and pathologic parameters in predicting disease progression, we sought to investigate the prognostic value of promoter methylation of a small panel of genes by quantitative methylation-specific PCR (QMSP) in prostate biopsies.

Experimental Design: Promoter methylation levels of *APC, CCND2, GSTP1, RARB2*, and *RASSF1A* were determined by QMSP in a prospective series of 83 prostate cancer patients submitted to sextant biopsy. Clinicopathologic data [age, serum prostate-specific antigen (PSA), stage, and Gleason score] and time to progression and/or death from prostate cancer were correlated with methylation findings. Log-rank test and Cox regression model were used to identify which epigenetic markers were independent predictors of prognosis.

Results: At a median follow-up time of 45 months, 15 (18%) patients died from prostate cancer, and 37 (45%) patients had recurrent disease. In univariate analysis, stage and hypermethylation of *APC* were significantly associated with worse disease – specific survival, whereas stage, Gleason score, high diagnostic serum PSA levels, and hypermethylation of *APC, GSTP1*, and *RASSF1A* were significantly associated with poor disease-free survival. However, in the final multivariate analysis, only clinical stage and high methylation of *APC* were significantly and independently associated with unfavorable prognosis, i.e., decreased disease-free and disease-specific survival.

Conclusions: High-level *APC* promoter methylation is an independent predictor of poor prognosis in prostate biopsy samples and might provide relevant prognostic information for patient management.

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Prostate cancer (PCa) is a highly prevalent malignancy and constitutes a major cause of cancer-related morbidity and mortality, accounting for 33% of all cancers diagnosed and for nearly 10% of all cancer deaths in U.S. males (1). Although serum prostate-specific antigen (PSA) is generally recommended for PCa screening, confirmation of diagnosis requires a prostate biopsy (2). The relevance of this biopsy exceeds its diagnostic purposes because the assessment of tumor grade and extent has a substantial impact on therapeutic decision making. However, the information provided by prostate biopsy meets with important limitations owing to intra- and interobserver variability in Gleason grading and sampling error (3, 4). Moreover, other accepted prognostic factors (e.g., clinical stage and pretherapeutic serum PSA levels) that influence treatment decisions are rather imperfect in predicting disease progression. The ability to predict disease-specific and disease-free survival at diagnosis is

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Table 1. Clinical and pathologic data from 83 PCa patients

ID*	Age	PSA (ng/mL)	Tumor content† (%)	GS	CS	Therapy	Follow-up data‡
1	77	52.0	≥75	7	II	ADT	Dec (9)
3	73	63.1	<25	7	III	ADT	Dec (43)
4	76	17.8	<25	6	II	EBRT	
5	67	7.0	≥75	7	III	PR	Rel (43)
7	51	4.5	25-50	6	II	PR	
10	64	5.4	<25	6	II	PR	
12	70	13.6	≥75	7	IV	EBRT	
14	71	11.6	<25	7	II	EBRT	Rel (51)
18	67	13.4	<25	6	II	PR	
20	62	38.2	<25	7	IV	ADT	Rel (30)
21	65	13.2	<25	6	II	PR	
23	78	22.6	50-75	7	II	ADT	Dec (45)
27	68	46.1	25-50	6	II	EBRT	
28	64	52.1	≥75	7	IV	ADT	Rel (33)
31	68	24.0	50-75	6	II	EBRT	
32	73	15.7	≥75	6	IV	ADT	Dec (41)
34	75	7.7	<25	6	II	EBRT	
35	79	62.6	<25	6	III	ADT	
36	68	35.8	≥75	6	II	ADT	Rel (57)
37	67	10.0	≥75	6	II	PR	
38	72	21.0	25-50	8	II	ADT	Rel (49)
39	79	43.9	≥75	8	IV	ADT	Rel (27)
41	57	12.0	<25	7	II	PR	Rel (17)
43	62	7.5	≥75	6	II	PR	
45	73	8.0	<25	6	II	ADT	Dec (13)
48	67	11.4	25-50	7	II	PR	
50	76	10.1	≥75	6	II	ADT	Rel (29)
52	69	7.1	<25	5	II	EBRT	
53	62	9.1	<25	6	II	PR	
54	68	17.9	≥75	7	II	EBRT	
56	67	14.5	≥75	6	II	EBRT	
57	68	36.3	≥75	7	II	EBRT	
58	69	9.6	≥75	7	II	PR	
59	80	19.2	<25	8	III	ADT	
61	66	18.5	≥75	7	II	ADT	Dec (16)
62	61	136.0	<25	7	III	ADT	Rel (51)
64	57	500.0	50-75	8	IV	ADT	Dec (46)
65	65	542.0	50-75	8	IV	ADT	
66	62	21.5	≥75	7	III	EBRT	Rel (29)
68	80	570.0	25-50	8	IV	ADT	
72	71	8.3	<25	7	II	PR	
73	66	14.0	<25	6	II	EBRT	
75	66	154.0	50-75	9	IV	ADT	Dec (28)
77	73	8.2	25-50	8	III	EBRT	Rel (29)
78	71	11.8	<25	6	II	EBRT	
79	80	15.8	<25	6	IV	ADT	Dec (46)
80	77	14.6	25-50	6	II	ADT	Rel (37)
83	70	8.6	≥75	7	II	EBRT	
84	75	77.9	25-50	8	III	ADT	Dec (29)
85	72	17.5	50-75	7	III	EBRT	
86	68	30.6	≥75	6	IV	ADT	Rel (45)
87	78	89.3	50-75	7	II	ADT	
88	71	8.8	<25	6	II	PR	
90	67	7.4	≥75	7	III	EBRT	
91	60	64.5	25-50	7	III	ADT	
94	70	45.0	≥75	7	II	ADT	Rel (32)
96	79	55.5	≥75	7	III	ADT	Dec (34)
98	72	12.4	≥75	6	II	EBRT	
100	60	11.4	<25	6	II	PR	
103	81	43.0	25-50	7	II	ADT	Rel (21)
105	62	12.6	≥75	7	II	PR	
106	67	14.2	50-75	6	II	PR	
107	75	10.7	≥75	6	II	ADT	Dec (42)
108	70	64.0	25-50	7	III	ADT	Dec (30)
110	71	30.8	≥75	6	II	ADT	

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Table 1. Clinical and pathologic data from 83 PCa patients (Cont'd)

ID*	Age	PSA (ng/mL)	Tumor content † (%)	GS	CS	Therapy	Follow-up data ‡
112	69	5.3	25-50	7	II	EBRT	
113	85	48.6	50-75	9	II	ADT	
115	80	287.0	≥75	6	IV	ADT	
119	71	11.0	50-75	8	II	PR	
120	73	89.5	25-50	7	IV	ADT	Rel (34)
125	64	10.9	≥75	8	II	EBRT	
130	57	520.0	<25	9	IV	ADT	Dec (9)
131	76	40.6	50-75	7	IV	ADT	Dec (37)
132	58	454.0	25-50	9	IV	ADT	
135	66	42.7	50-75	7	II	EBRT	Rel (21)
136	69	109.3	<25	6	III	ADT	Rel (25)
138	64	14.8	50-75	6	II	PR	
140	77	19.3	<25	7	III	ADT	
145	61	22.8	≥75	6	II	ADT	Rel (39)
149	78	39.9	<25	6	II	ADT	Rel (36)
150	65	5.4	<25	7	II	PR	Rel (54)
152	62	7.9	<25	6	II	PR	

Abbreviations: ADT, androgen deprivation therapy; EBRT, external-beam radiotherapy; PR, prostatectomy; GS, Gleason score; CS, clinical stage.
*Molecular cytogenetic findings in parallel biopsy samples taken from some of these patients have been previously reported (9), and the current sample IDs correspond to those published.
† Estimated tumor content upon morphologic analysis.
‡ Only death from disease events (Dec) and clinical relapse events (Rel) are displayed, along with the months at which the event occurred.

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particularly relevant in PCa patients, as any therapeutic option implies severe side effects. Thus, new and more accurate biological markers of prognosis are warranted for PCa patients.

Over the last decade, a new generation of cancer markers based on the characterization of epigenetic alterations, mainly promoter methylation, has emerged (5). We and others have reported on the value of quantitative assessment of promoter methylation of several cancer-related genes for discrimination between cancerous and noncancerous prostate tissues, allowing for the development of new early-detection strategies for PCa (reviewed in ref. 6). However, reports on the prognostic value of these epigenetic markers in tissue samples are rather scarce and are restricted to the evaluation of retrospective series of clinically localized cancers, which just represent a fraction of all prostate malignancies (7, 8).

We sought to investigate the prognostic value of promoter methylation of a panel of genes previously reported to be associated with clinicopathologic parameters of PCa progression (*APC, CCND2, GSTP1, RARB2*, and *RASSF1A*) and evaluated a model of prognosis prediction based on epigenetic alterations. For that purpose, quantitative methylation-specific PCR (QMSP) was used in a prospectively collected series of prostate biopsies from PCa suspects. Because we have previously shown that 8q gain detected in biopsies from PCa

Table 2. Correlations between clinical and epigenetic data in biopsy samples from 83 PCa patients

	Gleason score			*P*
	4-6 (*n* = 35)	7 (*n* = 34)	8-10 (*n* = 14)	
Age (y), median (range)	68 (51-80)	70 (57-81)	72 (57-85)	ns
PSA (ng/mL), median (range)*	14.0 (4.5-287.0)	22.0 (5.3-136.0)	63.3 (8.2-570.0)	0.003
Methylation levels, median (range)*				
APC	88.2 (0.0-802.5)	230.8 (0.2-1002.5)	170.4 (0.0-831.7)	0.003
CCND2	45.1 (0.5-915.7)	149.8 (0.0-525.7)	55.0 (0.0-391.3)	ns
GSTP1	94.0 (0.0-588.3)	207.3 (0.0-551.3)	208.5 (12.7-678.0)	0.018
RARB2	239.9 (0.5-1306.9)	408.5 (0.3-926.5)	288.9 (20.2-733.2)	ns
RASSF1A	242.7 (15.5-485.3)	350.4 (21.1-663.4)	268.8 (53.5-588.6)	0.011
High-level methylation frequency, (%)				
APC (levels >304.42)	2 (6)	15 (44)	4 (29)	0.001
CCND2 (levels >161.84)	4 (11)	15 (44)	2 (14)	0.004
GSTP1 (levels >292.82)	5 (14)	11 (32)	5 (36)	ns
RARB2 (levels >521.06)	8 (23)	10 (29)	3 (21)	ns
RASSF1A (levels >399.75)	4 (11)	14 (41)	3 (21)	0.016

Abbreviation: ns, not significant.
*Statistical comparisons involving PSA levels or methylation levels were done using nonparametric tests. Median values for each category are therefore displayed for informative purposes only.

patients was associated with worse disease outcome (9), we further analyzed all genomic (molecular cytogenetic and epigenetic) data available in the same set of biopsies.

Materials and Methods

Patients and samples. A series of 83 consecutive prostate biopsy specimens harboring prostate carcinoma was prospectively collected at the Portuguese Oncology Institute–Porto from individuals referred to this institution due to elevated PSA levels. In addition to the standard diagnostic cores, two supplementary tissue samples were collected from the more suspicious areas and frozen at -80°C. Sections of 5 μm were then cut, stained, and evaluated by a pathologist (R.H.) to assess the presence of neoplastic cells, after which the whole core was sectioned. DNA was extracted from all samples with phenol/chloroform and precipitated with ethanol (10). Histologic slides from formalin-fixed, paraffin-embedded tissue fragments were assessed for Gleason score (GS; ref. 11). Relevant clinical data at diagnosis, such as age, serum PSA level at diagnosis, clinical stage (CS; tumor-node-metastasis staging system ref. 12), therapeutic, and follow-up data, were obtained from medical records and are provided in Table 1, as well as the estimated tumor content of each biopsy core. The primary clinical end point of this study was disease-specific survival. Biochemical recurrence (considered when two consecutive PSA measurements above 0.4 ng/mL were obtained after complete PSA remission) was the secondary clinical end point of this study. Disease-specific and disease-free survival curves were computed according to the clinical, pathologic, and epigenetic parameters.

Bisulfite treatment and QMSP. Sodium bisulfite conversion of genomic DNA was done as previously described (13). The modified DNA was used as a template for real-time fluorogenic QMSP. The primers and probes used for the each target gene (i.e., *APC, CCND2, GSTP1*, *RARB2*, and *RASSF1A*) and for the internal reference gene (*β-actin, ACTB*) are listed elsewhere (13–17). To determine the relative levels of methylated promoter DNA in each sample, the values of each target gene were normalized against the values of the internal reference gene to obtain a ratio that was then multiplied by 1,000 for easier tabulation (target gene/*ACTB* ×1,000).

Fluorogenic QMSP assays were carried out in a reaction volume of 20 μL in 384-well plates in an Applied Biosystems 7900 Sequence Detector (Perkin-Elmer). PCR was done in separate wells for each primer/probe set, and each sample was run in triplicate. The final reaction mixture consisted of 600 nmol/L of each primer (Invitrogen); 200 nmol/L probe (Applied Biosystems); 0.75 unit of platinum Taq polymerase (Invitrogen); 200 μmol/L each of dATP, dCTP, dGTP, and dTTP; 16.6 mmol/L ammonium sulfate; 67 mmol/L Trizma; 6.7 mmol/L magnesium chloride; 10 mmol/L mercaptoethanol; 0.1% DMSO, and 3 μL bisulfite-converted genomic DNA. PCR was done using the following conditions: 95°C for 2 min, followed by 50 cycles at 95°C for 15 s and 60°C for 1 min.

Each plate included multiple water blanks, a negative control, and serial dilutions of a positive control for constructing the corresponding calibration curve. Leukocyte DNA collected from healthy individuals was used as negative control. The same leukocyte DNA was methylated *in vitro* with *Sss*I bacterial methyltransferase (New England Biolabs Inc.) and used as positive control for all studied genes. A given sample was considered positive when amplification was detected in at least two of the triplicates of the respective QMSP analysis. The QMSP threshold was determined for each run adjusting the best fit of the slope and R^2 based on the respective calibration curve.

Statistical analysis. For statistical purposes, prostate carcinoma samples were divided into three grade categories (GS ≤ 6, GS = 7, and GS ≥ 8). Clinical stage at diagnosis comprised three categories (stage II, III, and IV). Patients were also categorized by age groups (<65 versus ≥65 years) and according to PSA levels (<10 ng/mL versus ≥10 ng/mL). Clinical variables were compared for these groups using the χ^2 test, Fisher's exact test, or Kruskal-Wallis nonparametric test, according to the categorization of the variables. Differences in methylation levels for the five genes studied were assessed, within the different groups of clinical variables, using the Mann-Whitney and Kruskal-Wallis nonparametric tests according to the number of categories.

The prognostic significance of available clinical variables (CS, GS, age, and PSA) was assessed by constructing disease-specific and disease-free survival curves using the Kaplan-Meier method with log-rank test (univariate test) and by a Cox-regression model comprising all four variables (multivariate test). To test the prognostic significance of the methylation status for each gene, samples were categorized into two groups based on the methylation levels for that gene [using as a threshold the value of the percentile 75; ref. 8]. Disease-specific and disease-free survival curves were then constructed based on each of the five genes (univariate analysis). A Cox-regression model comprising both clinical and epigenetic variables was computed to assess the relative contribution of each variable to the assessment of follow-up status. A *P* value smaller than 0.05 (two-sided) was considered to indicate statistical significance. All analyses were done using SPSS version 11.0 (SPSS).

Results

Correlations between epigenetic data and clinicopathologic features. Relevant clinicopathologic and epigenetic data on the 83 biopsy patients is summarized in Table 2, whereas the

Table 2. Correlations between clinical and epigenetic data in biopsy samples from 83 PCa patients (Cont'd)

Clinical stage			*P*
II (*n* = 51)	**III (*n* = 16)**	**IV (*n* = 16)**	
68 (51-85)	73 (60-80)	69 (57-80)	ns
12.6 (4.5-89.3)	59.1 (7.0-136.0)	70.8 (13.6-570.0)	<0.001
124.1 (0.0-1002.5)	226.4 (0.8-536.1)	193.1 (0.0-831.6)	ns
79.8 (0.0-915.7)	138.9 (0.0-525.7)	46.7 (0.0-391.3)	ns
117.6 (0.0-588.3)	291.5 (0.0-551.3)	195.1 (13.9-677.9)	0.028
280.4 (0.5-926.5)	499.5 (0.3-1306.9)	277.9 (11.4-733.2)	ns
258.9 (15.5-663.4)	361.8 (27.9-587.5)	276.1 (153.6-588.6)	ns
12 (24)	6 (38)	3 (19)	ns
13 (26)	5 (31)	3 (19)	ns
9 (18)	8 (50)	4 (25)	0.034
11 (22)	8 (50)	2 (13)	ns
13 (26)	6 (38)	2 (13)	ns

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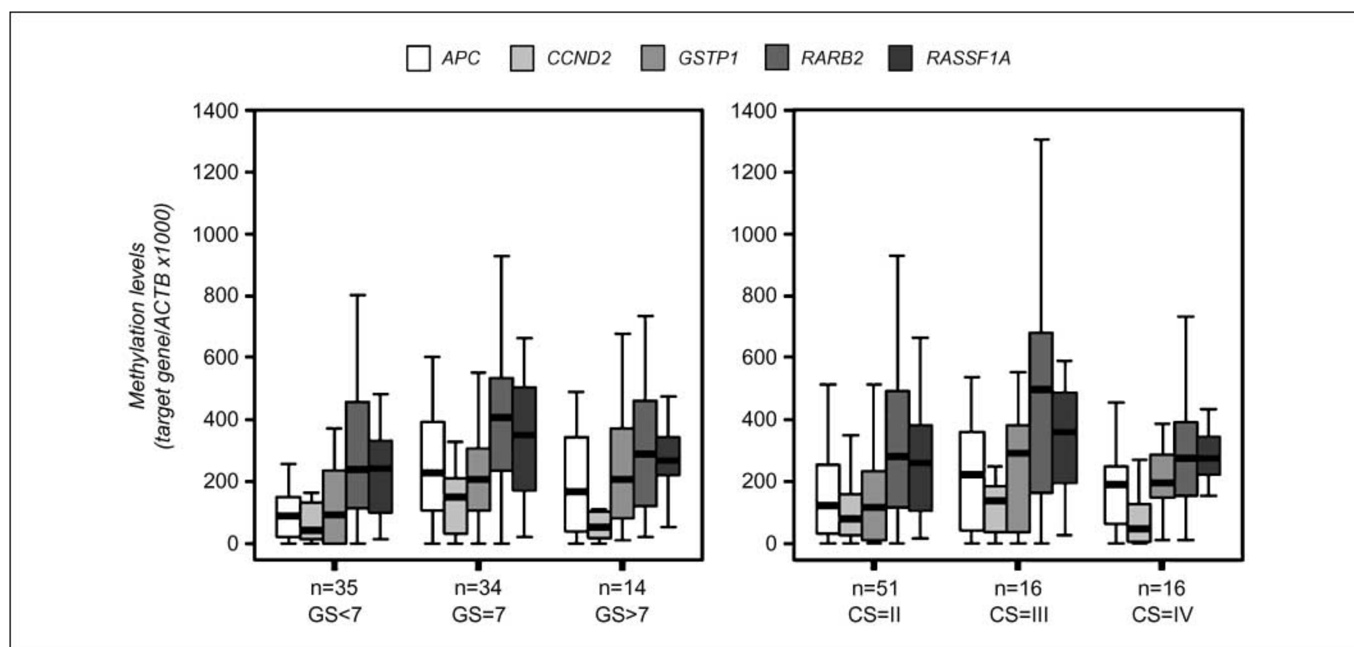

Fig. 1. Promoter methylation levels of five genes in biopsy samples from 83 PCa patients according to Gleason score and clinical stage categories (see Table 2 for the results of the nonparametric tests applied to this data). For visualization purposes only, outliers and extremes were filtered out of the box-plots. GS, Gleason score; CS, clinical stage.

distribution of the methylation levels along each stage and grade category is displayed in Fig. 1. Kruskall-Wallis nonparametric test showed that only the methylation levels of *GSTP1* were significantly different among clinical stage categories ($P = 0.028$), whereas increasing methylation levels in *GSTP1* ($P = 0.018$), *APC* ($P = 0.003$), and *RASSF1A* ($P = 0.011$) were associated with increased tumor grade (Table 2). When cases were coded based on methylation levels (using as a threshold the value of percentile 75 for each gene), the frequency of highly methylated samples for *APC* ($P = 0.001$), *CCND2* ($P = 0.004$), and *RASSF1A* ($P = 0.016$) increased significantly along GS categories, whereas that of *GSTP1A* ($P = 0.034$) was significantly higher in advanced-stage tumors (Table 2).

Survival analyses. The median follow-up of this series of patients was 45 months (range, 6-60 months). A total of 15 patients (18%) died from PCa during this period. Disease-specific survival curves using established clinical variables showed that advanced clinical stage was significantly associated with a worse outcome ($P = 0.016$), whereas GS, age, and PSA did not show prognostic value within the available follow-up time (Fig. 2 and Supplementary Fig. S1). Concerning epigenetic variables, high-level methylation of *APC* was the only event significantly associated with a worse disease–specific survival ($P = 0.010$, Fig. 2 and Supplementary Fig. S1). Interestingly, the prognostic value of *APC* methylation was independent from that of clinical variables, as both CS and *APC* methylation status were selected in the final Cox-regression model as significant predictors of disease-specific survival (Table 2).

Biochemical recurrence was detected in 37 (45%) patients during the follow-up period. Advanced clinical stage ($P = 0.002$), increased GS ($P = 0.007$), and diagnostic PSA levels above 10 ng/mL ($P = 0.033$) were all significantly associated with disease relapse in univariate analysis (Fig. 3 and Supplementary Fig. S2), whereas in multivariate analysis encompassing all clinical variables, only clinical stage surfaced as an independent prognostic indicator. Regarding epigenetic markers, high-level methylation of *APC* ($P = 0.002$), *GSTP1* ($P = 0.047$), or *RASSF1A* ($P = 0.019$) was significantly associated with a shorter time to relapse in univariate analysis (Fig. 3 and Supplementary Fig. S2). When clinical and epigenetic variables were introduced in a Cox-regression model for the prediction of relapse, both clinical stage and *APC* methylation levels were selected in the final step of the model as independent predictors (Table 3).

Because this series comprised patients subjected to different therapeutic strategies, determined mainly according to their age, serum PSA levels at diagnosis, Gleason score, and clinical stage, we did additional statistical analyses to determine *APC* hypermethylation prognostic value in each treatment group [i.e., radical prostatectomy (PR), external-beam radiation therapy (EBRT), and androgen deprivation therapy (ADT)]. Concerning disease-specific survival, there was a trend toward worse outcome for patients treated with ADT and *APC* hypermethylation ($P = 0.079$). In the remaining two treatment groups, no patient had yet deceased from PCa. Regarding disease-free survival, *APC* hypermethylation was associated with shorter time to biochemical relapse in the PR group of patients ($P = 0.043$), but no significant differences were found within the groups of patients treated with EBRT or ADT (Supplementary Fig. S3).

In a previous study from our group, which included 55 of the cases analyzed herein, we showed that 8q gain was associated with worse disease outcome, even after stratification according to tumor grade or clinical stage (9). Thus, we analyzed the cytogenetic and epigenetic data available in this set of biopsies. Interestingly, when gain of 8q was assessed in combination with *APC* hypermethylation, patients with at least one of these events were more prone to relapse ($P = 0.038$). However, no

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statistically significant result was found when disease-specific survival was the end point.

Discussion

In our previous studies, we identified a set of epigenetic alterations that correlated with clinical and pathologic features of tumor aggressiveness in PCa patients (13, 17, 18). Here, we present new data concerning the prognostic value of quantitative promoter methylation of a defined panel of cancer-related genes in a prospectively collected series of sextant prostate biopsies. The results we obtained concerning the prognostic significance of methylation-based markers in prostate biopsies should be addressed considering that the patient population theoretically includes the whole spectrum of PCa from the peripheral zone, ranging from indolent to

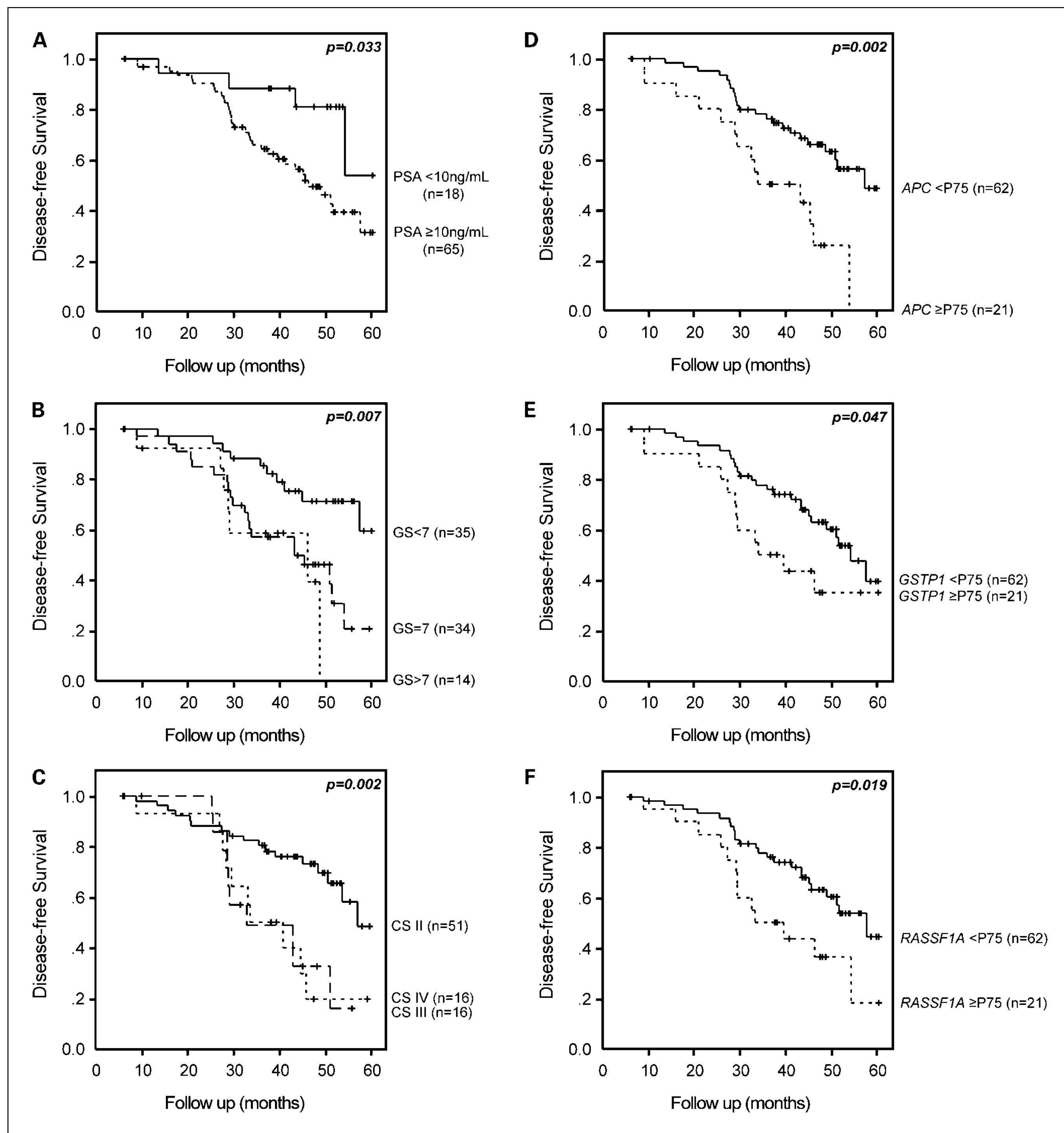

Fig. 2. Disease-free survival curves based on clinical variables (*A-C*) or epigenetic markers (*D-F*) in biopsy samples from 83 PCa patients (only statistically significant curves are shown, see Supplementary Fig. S1 for all variables tested). GS, Gleason score; CS, clinical stage; P75, percentile 75 of promoter methylation level.

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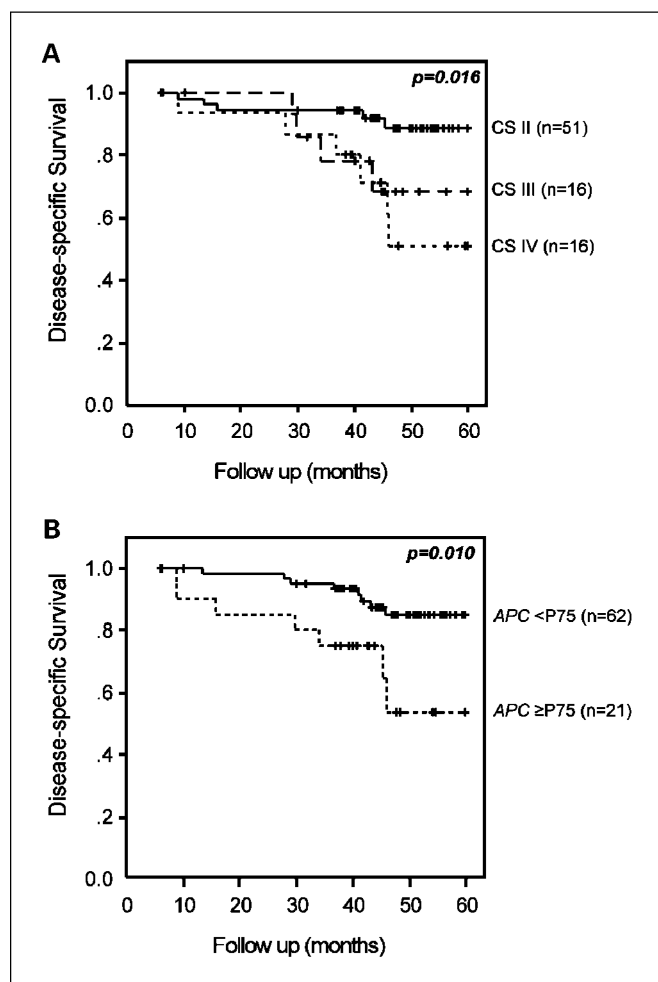

Fig. 3. Disease-specific survival curves based on clinical stage (*A*) and *APC* high-methylation levels (*B*) in biopsy samples from 83 PCa patients (see Supplementary Fig. S2 for all variables tested). GS, Gleason score; CS, clinical stage; P75, percentile 75 of promoter methylation level.

aggressive disease. Thus, direct comparisons with the scarce literature correlating PCa outcome and epigenetic alterations are not always feasible. Moreover, because follow-up data are still relatively short, some associations might not be statistically apparent, and this might constitute a limitation for our conclusions. However, owing to the novelty of the approach and the potential impact of some of the results, we believe that this preliminary data constitute an auspicious baseline for future research studies.

These conditions might explain the significant predictive power of clinical stage but not of the GS for disease-specific survival. However, it should be stated here that the GS only attains prognostic significance after 5 years of follow-up (19), which is the upper limit of our current follow-up data. It is expected that the predictive value of the GS becomes apparent in our series in the forthcoming reevaluation of the clinical data. Therefore, the predictive value of *APC* hypermethylation at 60 months of follow-up is a rather significant observation, considering the clinical emphasis placed in GS for therapeutic stratification of PCa patients. Indeed, although this epigenetic alteration is correlated with GS, it also displays an independent predictive value in multivariate analysis, confirming its potential clinical usefulness. To the best of our knowledge, this is the first report of an epigenetic marker that is able to predict preoperatively the disease-specific survival of PCa patients.

Because in many studies, biochemical recurrence is a primary end point, we also tested the predictive value of methylation-based markers in this setting. Remarkably, from the five genes in the panel, only *CCND2* did not show a significant association with disease-free survival in univariate analysis. However, only *APC* retained statistical significance in multivariate analysis. This result might be partially explained by the associations between promoter methylation levels of some genes and clinicopathologic parameters, i.e., clinical stage and GS, which were also predictors of poor survival in univariate analysis. Nevertheless, hypermethylation of *APC* confirmed its good performance as a prognostic factor in PCa patients for disease-free survival. Interestingly, it was previously reported that *APC* hypermethylation was a significant predictor for time to progression in a defined subset of PCas, i.e., GS 3 + 4 tumors (8). Our results extend this observation not only in GS range, but also in clinical stage because the tumors analyzed by Rosenbaum et al. (8) were all clinical stage II and, therefore, amenable to surgical resection. It should also be emphasized that *APC* hypermethylation has been reported as a promising marker for PCa detection in tissue samples (18, 20–22). Thus, determination of *APC* promoter methylation levels in prostate biopsies might not only constitute a valuable ancillary tool for PCa diagnosis, but it may also convey relevant prognostic information.

Because methylation levels depend on the DNA input of each sample (in this case the prostate biopsy core), we normalized

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Table 3. Cox regression models assessing the potential of clinical and epigenetic variables in the prediction of disease-specific or disease-free survival for 83 PCa patients

Model tested	Variables*	OR	95% CI for OR	*P*
Disease-specific survival	CS = IV versus CS = II	5.03	1.53-16.51	0.008
	APC methylation ≥ P75	3.51	1.23-9.96	0.018
Disease-free survival	CS = III versus CS = II	2.66	1.19-5.94	0.017
	CS = IV versus CS = II	3.09	1.40-6.82	0.005
	APC methylation ≥ P75	2.58	1.29-5.16	0.008

Abbreviations: OR, odds ratio; CS, clinical stage; CI, confidence interval; P75, percentile 75 for methylation level.
*Only variables displaying independent prognostic information in the final step of each model (forward conditional setting) are displayed.

the methylation results using an internal reference gene (*ACTB*). Owing to the marked variation in the tumor cell content of biopsy cores, it might be hypothesized that higher methylation levels could merely reflect increased tumor content, thus requiring further normalization. However, it is noteworthy that among the five target genes tested, only high *APC* methylation levels retained independent prognostic value. If tumor content was the major determinant of methylation levels, then the remaining genes would likely follow a similar trend. Moreover, the distribution of methylation levels according to tumor content followed different nonlinear patterns for each of the five genes, implying that biological differences are likely accounting for the observed variation among samples. Furthermore, we found that tumor content was not associated with disease-free or disease-specific survival (data not shown), and no predictive value was apparent when this parameter was included in the Cox-regression model. Thus, no subsequent adjustment to the tumor content of the biopsies was done.

A model incorporating clinical stage and *APC* quantitative promoter methylation provided the best strategy to stratify patients with shorter time to progression. Remarkably, it was suggested that *GSTP1* promoter methylation might be a relevant prognostic serum biomarker for PCa recurrence following radical prostatectomy (7). Although the results of both studies are not directly comparable owing to the different disease spectra, our findings partially corroborate those observations, as *GSTP1* quantitative promoter methylation showed prognostic significance in univariate analysis. Because Bastian et al. only tested *GSTP1* methylation as a prognostic marker, no conclusions can be drawn concerning the performance of *APC* promoter methylation in that setting. However, because our series encompasses a wider spectrum of PCa patients, it is likely that the performance of *APC* hypermethylation as a serum biomarker for disease progression might also prove effective.

In previous studies from our group, we found that patients whose tumors displayed 8q gain were nine times more likely to have a poor outcome, and its prognostic significance was retained after stratification for tumor grade or clinical stage (9). The prognostic significance of 8q gain was further confirmed by fluorescence *in situ* hybridization in an independent series of paraffin-embedded biopsies with longer follow-up (23). When cytogenetic and epigenetic results were combined in the survival analysis, the presence of 8q gain or *APC* hypermethylation was associated with disease progression. The lack of statistically significant result for disease-specific survival might be explained by the lack of sufficient follow-up time, as the trend for the two groups of patients seems to indicate. Alternatively, the explanation might lie in the association of both alterations in the more aggressive set of tumors. Hence, the combined use of cytogenetic and epigenetic information might define a more powerful genomic biomarker, supporting further investigation on the integration of genomic data in disease progression assessment of PCa patients.

It should be emphasized that the results presented herein are not definitive, and further studies are planned to test our hypotheses in a larger set of prostate biopsies with longer follow-up. Importantly, both molecular cytogenetic as well as epigenetic biomarkers showed its predictive value in a pre-therapeutic setting and, thus, may provide important ancillary information for clinical decision making.

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