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ORIGINAL REPORT

Promoter Methylation in *APC*, *RUNX3*, and *GSTP1* and Mortality in Prostate Cancer Patients

Lorenzo Richiardi, Valentina Fiano, Loredana Vizzini, Laura De Marco, Luisa Delsedime, Olof Akre, Anna Gillio Tos, and Franco Merletti

From the Cancer Epidemiology Unit, Center for Experimental Research and Medical Sciences and Center for Oncologic Prevention Piemonte, University of Turin; Department of Pathology, Molinette Hospital, Turin, Italy; and Clinical Epidemiology Unit, Karolinska Institutet, Stockholm, Sweden.

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Terms in blue are defined in the glossary, found at the end of this article and online at www.jco.org.

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Corresponding author: Lorenzo Richiardi, MD, PhD, Cancer Epidemiology Unit, University of Turin, Via Santena 7, 10126 Turin, Italy; e-mail: lorenzo.richiardi@unito.it.

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ABSTRACT

Purpose
There is a need to better understand prostate cancer progression and identify new prognostic markers for this tumor. We investigated the association between promoter methylation in a priori selected genes and survival in two independent large series of prostate cancer patients.

Methods
We followed up with two cohorts of patients (216 patients diagnosed in 1982 to 1988 and 243 patients diagnosed in 1993 to 1996) diagnosed at one hospital pathology ward in Turin, Italy. DNA was obtained from paraffin-embedded tumor tissues and evaluated for promoter methylation status in glutathione *S*-transferase (*GSTP1*), adenomatous polyposis coli (*APC*), and runt-related transcription factor 3 (*RUNX3*).

Results
The two cohorts had different prevalences of methylation in *APC* (P = .047), *GSTP1* (P = .002), and *RUNX3* (P < .001). Methylation in *APC* was associated with an increased risk of prostate cancer–specific mortality (hazard ratio [HR] = 1.42; 95% CI, 0.98 to 2.07 in the 1980s cohort; HR = 1.57; 95% CI, 0.95 to 2.62 in the 1990s cohort; HR = 1.49; 95% CI, 1.11 to 2.00 in the two cohorts combined). In subgroup analyses, the HRs were higher among patients with a Gleason score less than 8 (HR = 1.52; 95% CI, 0.85 to 2.73 in the 1980s cohort; HR = 2.09; 95% CI, 1.02 to 4.28 in the 1990s cohort). Methylation in *RUNX3* was associated with prostate cancer mortality only in the 1990s cohort, and methylation in *GSTP1* did not predict mortality in either cohort.

Conclusion
The pattern of hypermethylation may have changed after the introduction of prostate-specific antigen testing in the beginning of the 1990s. Promoter methylation in *APC* was identified as a marker for prostate cancer progression.

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INTRODUCTION

Prostate cancer is the most frequent cancer in the United States and Western Europe.[1] The incidence has been increasing by approximately 3% per year during several decades.[2] The trends shifted in the late 1980s and early 1990s when prostate-specific antigen (PSA) testing became widespread.[3] In the United States, for example, the annual percent change in incidence was 2.4% before 1989 and 18.4% between 1989 and 1992.[4]

The use of PSA testing remains under debate. First, PSA testing has low sensitivity and positive predictive value, implying high proportions of both false-positive and false-negative tests.[5,6] Second, PSA screening identifies indolent cancers, resulting in overdiagnosis.[7] Patients diagnosed with localized prostate cancers, notably those detected through PSA, may have an excellent prognosis when left untreated. For these patients, it would be important to distinguish between indolent and aggressive tumors. Several preoperative nomograms developed in the last 10 years have been based on clinicopathologic variables, including PSA, Gleason score, clinical stage, and number of positive and negative biopsy cores.[8,9] The collective prognostic value of these factors is unsatisfactory, and better understanding of the biology of prostate cancer progression is needed to identify new markers.[9,10]

Emerging evidence indicates that epigenetic alterations, particularly DNA hypermethylation, play a role in human carcinogenesis and tumor progression.[11,12] Several studies found that presence of CpG island (clusters of dinucleotides of a cytosine and a guanosine) methylation in the promoter of some cancer-related genes, such as glutathione *S*-transferase (*GSTP1*), adenomatous polyposis coli (*APC*), and PTGS2, may be

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used as a diagnostic test to distinguish between normal and prostate cancer tissue.[13,14] In addition, methylation in single genes and methylation indices have been found to be associated with clinicopathologic indicators of poor prognosis, although there is inconsistency between studies.[15-23] The association between promoter hypermethylation in prostate cancer and clinical outcome or mortality has been less investigated, and investigation was performed mostly in relatively small patient series with short follow-up using heterogeneous or intermediate outcomes.[15,18,24-27]

We studied the prostate cancer survival in association with promoter methylation in *GSTP1*, *APC*, and runt-related transcription factor 3 (*RUNX3*). *GSTP1* is the most frequently investigated gene in prostate cancer epigenetics and has been found to be frequently methylated in prostate tumor tissues in several studies. *APC* and *RUNX3* were selected among genes (*EDNRB*, *COX2*, *PTGS2*, *APC*, *RASSF1*, and *RUNX3*) for which aberrant methylation status was reported to be associated with clinical features of poor prognosis in prostate cancer patients at the time when our study was designed.[28] Because they are involved in signaling and transcription pathways, their inhibition by promoter methylation may plausibly have a role in prostate cancer progression.[28,29] In addition, their polymerase chain reaction (PCR) target sequences were short enough to be successfully investigated by published primers in paraffin-embedded tissues preserved for several years.

Two independent cohorts of, in total, 459 prostate cancer patients were observed for prostate cancer mortality. The first cohort included patients diagnosed in the 1980s, whereas members of the second cohort were diagnosed in the 1990s. The second cohort was used to validate the findings in the first cohort and to study any possible changes in the methylation patterns between the 1980s, before the introduction of PSA testing, and the 1990s, during the PSA era.

Table 1. Characteristics of the Two Cohorts of Prostate Cancer Patients After 14 Years of Follow-Up

Characteristic	1980s Cohort (1982-1988) No. of Patients	1980s Cohort (1982-1988) %	1990s Cohort (1993-1996) No. of Patients	1990s Cohort (1993-1996) %	P*
No. of patients	216		243		
No. of person-years	1,040		1,591		
Mortality					
Overall	195		177		
As a result of prostate cancer	121		76		
As a result of other causes	74		101		
Missing cause of death	8		0		
Survival time, years					
Median	3.1		6.3		
Range	0-14		0-14		
Age, years					.003
Mean	72.3		70.0		
Standard deviation	7.5		8.7		
Residence					.692
Turin	153	70.8	168	69.1	
Turin hinterland	63	29.2	75	30.9	
Source of tumor tissue					< .001
Biopsy	182	84.3	164	67.5	
TURP	11	5.1	45	18.5	
Radical prostatectomy	23	10.6	34	14.0	
Gleason score					< .001
< 7	32	14.8	136	56.0	
7	85	39.4	34	14.0	
8+	99	45.8	73	30.0	
Methylation in *GSTP1*					.002
Yes	159	76.1	150	62.8	
No	50	23.9	89	37.2	
Missing	7	—	4	—	
Methylation in *APC*					.047
Yes	76	35.9	106	45.1	
No	136	64.1	129	54.9	
Missing	4	—	8	—	
Methylation in *RUNX3*					< .001
Yes	170	84.6	103	48.1	
No	31	15.4	111	51.9	
Missing	15	—	29	—	

Abbreviation: TURP, transurethral resection of the prostate.
*P value for difference between 1980s cohort and 1990s cohort.

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METHODS

Cohorts

The cohorts consist of consecutive prostate cancer patients of any age identified at a single pathology ward of the San Giovanni Battista Hospital, the main hospital in the city of Turin (900,000 inhabitants), Italy. The first cohort, hereafter referred to as the 1980s cohort, includes patients who received a biopsy of the prostate, transurethral resection of the prostate, or radical prostatectomy between 1982 and 1988. Patients in the second cohort, the 1990s cohort, were diagnosed at the hospital between 1993 and 1996. The 1980s and 1990s cohorts included 298 and 280 eligible patients, respectively. The study was approved by the local ethical committee.

Slices of formalin-fixed and paraffin-embedded tumor tissue (PETs) were obtained from each patient. DNA extraction from the PETs was successful in 77% (n = 228) of the patients in the 1980s cohort and 90% (n = 253) of patients in the 1990s cohort. Patients with successful extraction remained for further analysis.

From each patient's pathology report, we obtained information on name and surname, age, tumor grade, place of residence, and, limited to the 1990s cohort, Gleason score. Three patients in the 1980s cohort and two patients in the 1990s cohort with incorrect demographic information were excluded from the study. Diagnostic slides for patients in the 1980s cohort were traced and re-evaluated by a pathologist (L.D.), who assigned the Gleason score. We could not trace the slides of eight tumors. In those cases, we used the information on tumor grade that was available in the pathology report; well-differentiated tumors were translated to a Gleason score of 6 or less, moderately differentiated tumors corresponded to a score of 7, and poorly differentiated tumors had a score of 8+.

Follow-Up

We observed the patients from the date of the pathology report to February 13, 2006 for the 1980s cohort and to January 15, 2007 for the 1990s cohort. Dead patients were censored on their date of death. Information on vital status and copies of the death certificates came from the demographic offices of Turin and the towns of the hinterland, and we ascertained migration at the Migration Office. Follow-up information was 95% complete (nine patients lost) for the 1980s cohort and 96% complete (eight patients lost) for the 1990s cohort. Patients with no follow-up information were excluded from the study. The death certificates for eight patients in the 1980s cohort were not retrievable. These patients were excluded from the analyses focusing on prostate cancer mortality.

Molecular Analyses

We extracted genomic DNA from 3 to 5 (10-μm thick) sequential sections of PETs and checked for adequacy by PCR amplification of the β-globin gene.[30] If a patient had multiple blocks of PET, a block embedding tissue with tumor cells was chosen after histologic review of the corresponding slide. If a patient had both biopsy and prostatectomy blocks, we analyzed the biopsy. For all patients retained in the study, β-globin PCR analysis resulted in clearly sharp detectable amplicons after gel electrophoresis, allowing adequacy for methylation-specific analyses.

The genomic DNA samples, including positive controls for methylated and unmethylated status, underwent bisulfite modification using CpGenome DNA Modification Kit (Intergen Co, Purchase, NY) following the manufacturer's instructions.[31] Bisulfite-modified DNA was used as a template for PCR amplification using primers specific for either the methylated or the modified unmethylated DNA. The sets of specific primers and their annealing temperatures for methylated and unmethylated forms of *GSTP1*, *APC*, and *RUNX3* gene promoters were selected from published sequences.[20,32] For PCR amplification, 4 μL of bisulfite-modified DNA was added in a final volume of 25 μL PCR mix containing 1X PCR buffer (15 mmol/L Tris, pH 8.0; 50 mmol/L KCl; and 6.7 mmol/L $MgCl_2$), deoxynucleotide triphosphates (2 mmol/L each), primers (0.4 μmol/L each per reaction), and 1.25 U of AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA). PCR conditions were as follows: 10 minutes at 95°C, 30 seconds at 95°C, 1 minute at primer-specific annealing temperature, 1 minute at 72°C for 45 cycles, and 7 minutes at 72°C.[20,32] All PCR amplifications were performed in a Gene Amp PCR System 9700 Thermal Cycler (Applied Biosystems). Bisulfite-modified CpGenome universal methylated DNA (Intergen Co) was used as positive control for methylated alleles, and bisulfite-modified DNA from normal human lymphocytes was used as a positive control for unmethylated alleles. Negative PCR controls without DNA were included in each PCR run. Ten microliters of each PCR amplification were loaded onto 2% agarose gel stained with ethidium bromide and visualized by ultraviolet transillumination. As shown in previous studies, this method has high sensitivity, detecting one methylated nucleotide in 1,000 unmethylated nucleotides.[33]

Statistical Analyses

We compared clinical and pathologic characteristics of the patients in the 1980s cohort and the 1990s cohort using univariate χ^2 and *t* tests.[34] Variables were classified as reported in Table 1. No information on PSA variables, tumor stage, and number of positive and negative biopsies was available in the pathology reports.

Through logistic regression, we estimated, separately for the 1980s and 1990s cohorts, the prevalence odds ratios for the association between methylation in the genes and clinical and pathologic characteristics.[35] Patients with missing methylation status in one gene were excluded from the corresponding analyses.

For the older cohort, we ended follow-up after 14 years to apply the same maximum follow-up for both cohorts. The effect of methylation status in each of the three genes on cumulative mortality from prostate cancer was investigated taking into account competing risks,[36] and differences in overall prostate

Table 2. Association Between Gleason Score and Prevalence of Methylation in *GSTP1*, *APC*, and *RUNX3* in 1980s and 1990s Cohorts

	GSTP1			*APC*			*RUNX3*		
Gleason Score	Prevalence of Methylation (%)	POR*	95% CI	Prevalence of Methylation (%)	POR*	95% CI	Prevalence of Methylation (%)	POR*	95% CI
1980s cohort									
< 7	65.5	1	—	25.8	1	—	89.3	1	—
7	82.1	2.27	0.81 to 6.34	40.5	1.66	0.64 to 4.31	81.0	0.40	0.10 to 1.70
8+	74.0	1.18	0.45 to 3.13	35.1	1.25	0.48 to 3.25	86.2	0.58	0.13 to 2.53
1990s cohort									
< 7	57.0	1	—	38.5	1	—	44.7	1	—
7	62.5	1.21	0.53 to 2.77	48.5	1.39	0.63 to 3.08	48.4	1.21	0.53 to 2.76
8+	73.6	2.02	1.06 to 3.84	55.6	2.07	1.13 to 3.81	53.6	1.36	0.73 to 2.52

Abbreviation: POR, prevalence odds ratio.
*POR adjusted for age, source of tumor tissue, year of tissue collection, and residence.

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cancer mortality were evaluated with the Gray's test.[37] Using age as the temporal axis, we used Cox proportional hazards regression models to estimate hazard ratios (HRs) with 95% CIs of prostate cancer mortality by methylation status and Gleason score (two categories: < 8 and ≥ 8). Patients were censored at death from causes other than prostate cancer. Both a graphical check and formal tests based on Schoenfeld residuals ($P > .15$) indicated that the proportional hazard assumption was met. We included the following covariates in the models: source of tumor tissue, Gleason score, and follow-up duration (time-dependent variable in three categories: < 1 year, between 1 and 3 years, and 3+ years). Inclusion of place of residence in the models did not affect HR estimates. HRs specific for categories of follow-up duration were estimated introducing terms for the interaction between this variable and methylation status.

We also investigated the interaction between Gleason score and methylation in *APC* and *RUNX3*. In exploratory analyses, we further studied the association between survival and number of methylated genes. Patients with missing methylation status in at least one of the genes were excluded from this analysis (n = 53, 12% of the patients).

To understand whether a lack of cancer cells in some tissue slices biased our estimates, we provisionally restricted survival analyses to patients positive for methylation in *GSTP1*, for whom tumor cells were most likely sufficiently represented, and patients who underwent biopsy, where all available tissue was paraffin embedded in one single block.

RESULTS

Patients in the 1990s cohorts were younger and had twice the median survival time than those in the 1980s cohort (Table 1). In the 1990s cohort, tumor tissue was obtained from transurethral resections of the prostate and radical prostatectomies more often than in the 1980s cohort.

Prevalence of Promoter Methylation

In the 1980s cohort, the prevalence of methylation in *APC* was lower ($P = .047$) but methylation in *GSTP1* ($P = .002$) and *RUNX3* ($P < .001$) was more frequent compared with the 1990s cohort. These differences remained after adjustment for Gleason score, age, and source of the tumor tissue.

Methylation in *GSTP1* and *APC* was positively associated with Gleason score only in the 1990s cohort (Table 2). Age, source of tumor tissue, and year of tissue collection were not associated with methylation (data not shown). In both cohorts, pair-wise comparisons revealed that methylation in each gene was independent from methylation in the other two genes ($P > .25$).

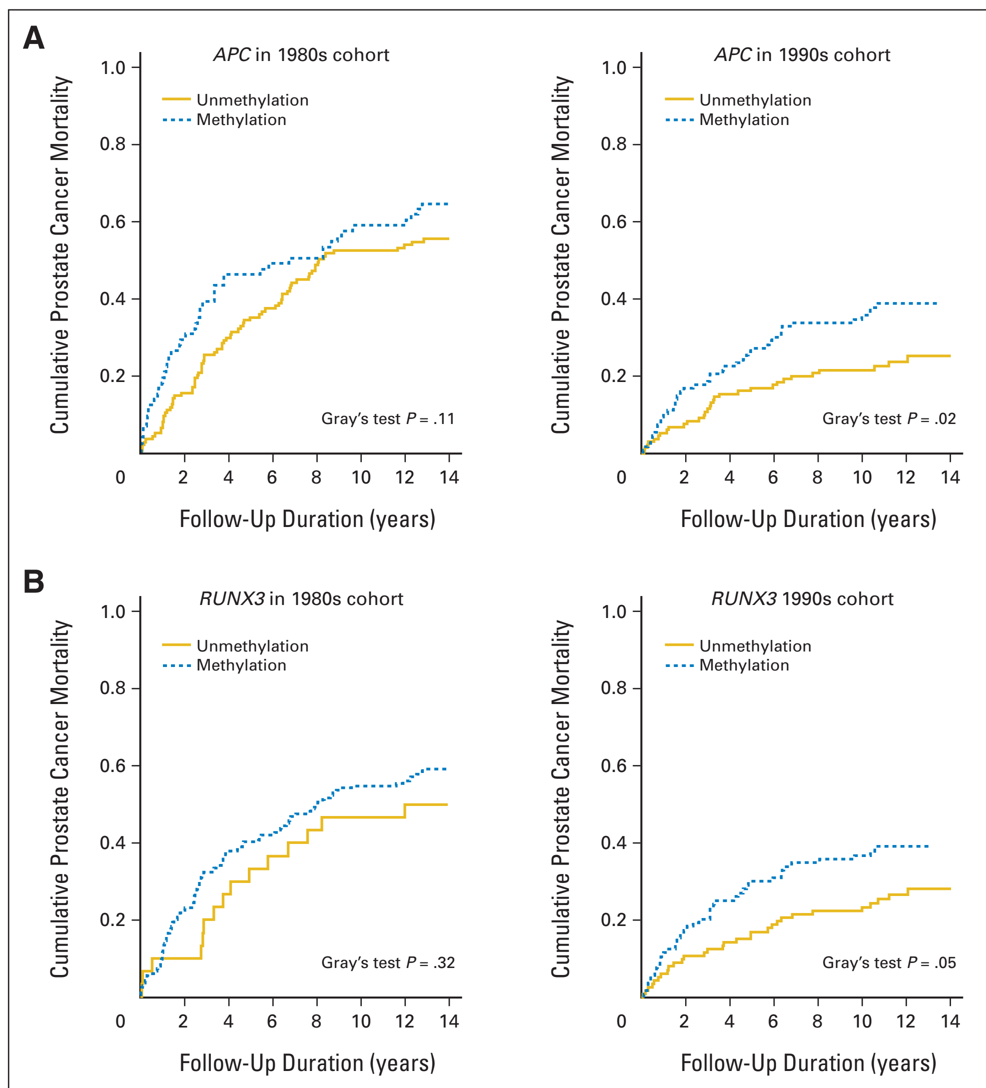

Fig 1. Cumulative prostate cancer mortality by methylation status in (A) *APC* and (B) *RUNX3* in the 1980s cohort and 1990s cohort.

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Promoter Methylation and Tumor Progression

Patients with methylation in *APC* had a higher prostate cancer mortality than patients with an unmethylated cancer (Fig 1A). This association was statistically significant only in the 1990s cohort (P = .02). Methylation in *RUNX3* was associated with survival in the 1990s cohort (P = .05) but not in the 1980s cohort (Fig 1B). Methylation in *GSTP1* was not associated with survival (data not shown).

The HR of prostate cancer mortality for methylation in *APC* was 1.42 (95% CI, 0.98 to 2.07) in the 1980s cohort and 1.57 (95% CI, 0.95 to 2.62) in the 1990s cohort (HR = 1.49; 95% CI, 1.11 to 2.00 for the two cohorts combined; Table 3). In the 1990s cohort, the adjusted HR estimate was lower than the crude one, mainly because of the introduction of Gleason score into the model. In the 1980s cohort, the association between methylation in *APC* and mortality was stronger and statistically significant in the first year of follow-up, whereas in the 1990s cohort, the HR estimate increased with duration of follow-up.

Results were not consistent between the two cohorts for methylation in *RUNX3*, which was associated with prostate cancer mortality in the 1990s cohort (HR = 1.56; 95% CI, 0.95 to 2.56) but not in the 1980s cohort (Table 3). Methylation in *GSTP1* was not associated with prostate cancer mortality. Restriction of the analyses to patients who underwent biopsy or were positive for *GSTP1* methylation did not substantially change increased HR estimates for methylation in *APC* and *RUNX3* (data not shown).

The effect of methylation in *APC* or *RUNX3* at different levels of Gleason score is summarized in Table 4. In the 1980s cohort, Gleason score had a small impact on the HR estimates, whereas in the 1990s cohort, we found a doubled HR from prostate cancer mortality among patients with a Gleason score less than 8 both for methylation in *APC* (HR = 2.09; 95% CI, 1.02 to 4.28) and in *RUNX3* (HR = 2.40; 95% CI, 1.18 to 4.91). In the two cohorts combined, the HR of prostate cancer mortality increased with increasing number of methylated genes (P = .002 for linear trend; Table 5).

DISCUSSION

We found that methylation in *APC* is associated with prostate cancer mortality, particularly among those with a highly to moderately differentiated tumor. A similar association was found for methylation in *RUNX3* in the 1990s cohort, whereas methylation in *GSTP1* was not associated with risk. The results also indicated a shift in the methylation patterns from the 1980s to the 1990s.

Table 3. Prostate Cancer Mortality for Methylation in *GSTP1*, *APC*, and *RUNX3* by Duration of Follow-Up in 1980s and 1990s Cohorts

Methylation and Gleason Score	No. of Deaths	Overall 14-Year Follow-Up			Follow-Up Period: < 1 Year		Follow-Up Period: 1-3 Years		Follow-Up Period: 3+ Years	
		Crude HR	HR*	95% CI	HR*	95% CI	HR*	95% CI	HR*	95% CI
1980s cohort										
Methylation in *GSTP1*										
No	26	1	1		1		1		1	
Yes	89	1.08	1.00	0.64 to 1.58	0.42	0.17 to 1.03	0.94	0.41 to 2.12	1.64	0.79 to 3.41
Methylation in *APC*										
No	74	1	1		1		1		1	
Yes	46	1.46†	1.42	0.98 to 2.07	2.66	1.12 to 6.31	1.41	0.72 to 2.75	1.10	0.63 to 1.93
Methylation in *RUNX3*										
No	15	1	1		1		1		1	
Yes	97	1.32	1.22	0.70 to 2.14	0.97	0.27 to 3.43	2.39	0.72 to 7.89	0.93	0.45 to 1.94
Gleason score										
< 8	50	1	1		1		1		1	
8+	71	2.53†	2.17	1.48 to 3.18	3.39	1.29 to 8.91	1.87	0.97 to 3.60	2.07	1.21 to 3.52
1990s cohort										
Methylation in *GSTP1*										
No	17	1	1		1		1		1	
Yes	58	2.02†	1.44	0.82 to 2.54	1.62	0.52 to 5.04	0.94	0.34 to 2.62	1.74	0.75 to 4.04
Methylation in *APC*										
No	31	1	1		1		1		1	
Yes	41	1.99†	1.57	0.95 to 2.62	1.48	0.55 to 3.96	1.15	0.42 to 3.16	1.86	0.93 to 3.72
Methylation in *RUNX3*										
No	30	1	1		1		1		1	
Yes	40	1.74	1.56	0.95 to 2.56	1.58	0.60 to 4.14	1.33	0.48 to 3.68	1.66	0.85 to 3.27
Gleason score										
< 8	36	1	1		1		1		1	
8+	36	3.50†	3.27	2.00 to 5.37	6.50	2.08 to 20.3	3.61	1.31 to 9.94	2.34	1.16 to 4.73

Abbreviation: HR, hazard ratio.
*HR was adjusted for follow-up duration, Gleason score, and source of tumor tissue; Gleason score was also adjusted for methylation in *APC*.
†$P < .05$.

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Table 4. Prostate Cancer Mortality for Gleason Score Strata and Methylation in *APC* and *RUNX3* in 1980s and 1990s Cohorts

	Gleason < 8			Gleason 8+		
Methylation	No. of Deaths	HR*	95% CI	No. of Deaths	HR*	95% CI
1980s cohort						
Methylation in *APC*						
No	30	1		44	1	
Yes	20	1.52	0.85 to 2.73	26	1.36	0.81 to 2.26
Methylation in *RUNX3*						
No	6	1		9	1	
Yes	38	1.14	0.45 to 2.88	59	1.09	0.52 to 2.29
1990s cohort						
Methylation in *APC*						
No	16	1		15	1	
Yes	20	2.09	1.02 to 4.28	21	1.07	0.51 to 2.26
Methylation in *RUNX3*						
No	14	1		16	1	
Yes	21	2.40	1.18 to 4.91	19	0.84	0.38 to 1.85

Abbreviation: HR, hazard ratio.
*HR was adjusted for follow-up duration and source of tumor tissue.

Gene-specific prevalences of methylation were different between the two cohorts. These differences are unlikely a result of selection of patients or laboratory heterogeneities for the following reasons: the same methodologies for molecular analyses were used in the two cohorts; within each cohort, the lack of association of the year of tissue collection with prevalence of methylation suggests that tissue preservation did not affect the results; both cohorts included an unselected series of patients, and the catchment area of the hospital did not change over time; and in the 1990s cohort, a higher number of patients received radical prostatectomy, but the differences between the two cohorts remained in the comparison restricted to patients who underwent biopsy.

The decrease in age at diagnosis and increase in survival that we observed in the 1990s cohort is consistent with an effect of opportunistic PSA screening.[7] It has been estimated that, each year, more than 10% of men older than 50 years received a PSA test at the end of the 1990s in Northern Italy.[38,39] Therefore, one plausible explanation for the observed difference in methylation prevalences between the 1980s cohort and the 1990s cohort could be that PSA-detected prostate cancers are characterized by a different methylation pattern. This could be explained either by a larger proportion of early tumors or a greater proportion of indolent cancers in the 1990s cohort. Distinguishing between these two mechanisms cannot be done with our data, but it would help in the understanding of the biology of prostate cancer and possibly in the identification of new prognostic markers.

Table 5. Prostate Cancer Mortality for Number of Methylated Genes in 1980s and 1990s Cohorts Combined

No. of Methylated Genes	No. of Deaths	HR*	95% CI
0	5	0.80	0.31 to 2.08
1	32	1.00	
2	94	1.53	1.02 to 2.30
3	44	1.97	1.24 to 3.15

Abbreviation: HR, hazard ratio.
*HR was adjusted for follow-up duration, Gleason score, source of tumor tissue, and cohort. $P = .002$ for trend.

The methylation prevalences that we found are in accordance with previous data. Prevalence of *GSTP1* methylation has been estimated to be greater than 60%, with large heterogeneities between studies.[13,28,40] The two largest studies conducted so far found prevalences of 73% in 179 patients[22] and 66% in 291 patients.[19] Most of the studies that investigated methylation in *APC* in prostate cancer found a prevalence of greater than 50%.[14,15,17,20,22,24,26,28,41-44] Little is known about the prevalence of methylation in *RUNX3* from previous studies.[20,22]

Our data suggest that methylation in *APC* may be involved in prostate cancer progression. Even if we lacked information on some important variables, such as PSA and TNM stage, residual confounding is unlikely to be a major limitation. First, we adjusted for Gleason score, which is a strong prognostic variable. Second, studies evaluating the correlation between methylation in *APC* and clinicopathologic variables found heterogeneous results, with most of the association estimates being weak.[14,17,22,43] The confounding potential of these variables after adjustment for Gleason score should thus be limited.

To date, little is known about the role of epigenetics and promoter methylation in prostate cancer progression. Our results on *APC* are predated by similar findings from two recent smaller studies. In a cohort of 74 prostate cancer patients who underwent prostatectomy, approximately 70% of the patients experienced PSA reoccurrence, metastasis, or death, with an HR for promoter methylation in *APC* of 3.0 (95% CI, 1.4 to 6.3).[24] A three-fold statistically significantly increased HR has also been reported by Henrique et al,[26] who investigated 83 prostate cancer patients, of whom 15 died from prostate cancer during follow-up.

The *APC* complex is known from studies of colorectal cancer cells to function as a gatekeeper in the cell, preventing the transcription of gene products that promote cell proliferation and survival rather than differentiation and apoptosis.[45] Hypermethylation of *APC* implies silencing of this gatekeeper, making the cell vulnerable to further

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epigenetic and genetic changes and, thus, progression toward the development of invasive cancer. This is consistent with our finding of a decreasing survival with number of methylated genes, which further supports a role of gene hypermethylation in cancer progression. We also found that hypermethylation of *APC* is associated with survival among patients with tumors of high to moderate differentiation but not among those with a poorly differentiated tumor. This finding may suggest that changes in *APC* might occur early in the tumorigenesis in the prostate, making the cell vulnerable to further changes, but in tumors with poor differentiation, changes in *APC* add little to the malignant potential.

In this large survival analysis of two independent series of unselected prostate cancer patients, we found that hypermethylation in the promoter of the *APC* gene is involved in prostate cancer progression. The possibility of using this as a prognostic marker will have to be addressed in future independent cohorts with more detailed clinical information available. When comparing patients from the 1980s, before the introduction of PSA testing, with those from the 1990s, after the introduction of PSA, we found a considerable change in the methylation pattern, possibly indicating different biologic behavior of PSA-detected and other prostate cancers.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

The author(s) indicated no potential conflicts of interest.

AUTHOR CONTRIBUTIONS

Conception and design: Lorenzo Richiardi, Anna Gillio Tos, Franco Merletti
Financial support: Lorenzo Richiardi, Franco Merletti
Provision of study materials or patients: Valentina Fiano, Laura De Marco, Luisa Delsedime
Collection and assembly of data: Valentina Fiano, Laura De Marco, Luisa Delsedime
Data analysis and interpretation: Lorenzo Richiardi, Valentina Fiano, Loredana Vizzini, Laura De Marco, Luisa Delsedime, Olof Akre, Anna Gillio Tos, Franco Merletti
Manuscript writing: Lorenzo Richiardi, Loredana Vizzini, Olof Akre, Anna Gillio Tos, Franco Merletti
Final approval of manuscript: Lorenzo Richiardi, Valentina Fiano, Loredana Vizzini, Laura De Marco, Luisa Delsedime, Olof Akre, Anna Gillio Tos, Franco Merletti

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Glossary Terms

Promoter hypermethylation: Methylation of the promoter region of a gene can lead to DNA silencing as a consequence of the inability of activating transcriptional factors to bind to the promoter region, a process important in gene transcription. In addition, repressor complexes may be attracted to sites of promoter methylation, leading to the formation of inactive chromatin structures.

CpG island: DNA sequences with a high density of CpGs are termed CpG islands. CpG islands are typically unmethylated in normal tissues but often become methylated in tumors. The patterns of hypermethylated CpG islands vary according to the histologic origin of the tumor.

Epigenetic: The transfer of information from one cell to its descendants without the information's being encoded in the nucleotide sequence of the DNA. The methylation of the promoter to inactivate a gene is an example of an epigenetic change. Epigenetic inheritance is typically transmitted in dividing cells. Although rare, it is occasionally seen in traits being transmitted from one generation to another. Epigenetic variants can arise spontaneously and just as spontaneously revert.

PSA (prostate-specific antigen): A protein produced by cells of the prostate gland, the blood level of PSA is used as a tumor marker for men who may be suspected of having prostate cancer. Most physicians consider 0 to 4.0 ng/mL as the normal range. Levels of 4 to 10 and 10 to 20 ng/mL are considered slightly and moderately elevated, respectively. PSA levels have to be complemented with other tests to make a firm diagnosis of prostate cancer.

APC (adenomatous polyptosis coli) gene: A tumor suppressor gene. Mutations in the gene are responsible for familial adenomatous polyposis (germline mutations) or sporadic (somatic mutations) colorectal tumors. The gene product is known to interact with adherens junction proteins, a- and b-catenins, suggesting a role in cell adhesion.

Cox proportional hazards regression model: The Cox proportional hazards regression model is a statistical model for regression analysis of censored survival data. It examines the relationship of censored survival distribution to one or more covariates. It produces a baseline survival curve, covariate coefficient estimates with their standard errors, risk ratios, 95% CIs, and significance levels.

PCR (polymerase chain reaction): PCR is a method that allows logarithmic amplification of short DNA sequences within a longer DNA molecule.

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