Aberrant Promoter Methylation Profile of Prostate Cancers and Its Relationship to Clinicopathological Features[1]

Riichiroh Maruyama, Shinichi Toyooka, Kiyomi O. Toyooka, Arvind K. Virmani, Sabine Zöchbauer-Müller, Alfredo J. Farinas, John D. Minna, John McConnell, Eugene P. Frenkel, and Adi F. Gazdar

Hamon Center for Therapeutic Oncology Research [R. M., S. T., K. O. T., A. K. V., S. Z-M., A. J. F., J. D. M., A. F. G.], the Departments of Pathology [A. K. V., A. F. G.], Internal Medicine [J. D. M., E. P. F.], Pharmacology [J. D. M.], and Urology [J. M.], and the Simmons Cancer Center [E. P. F.], University of Texas Southwestern Medical Center, Dallas Texas 75390

ABSTRACT

Purpose: **We investigated the aberrant methylation profile of prostate cancers and correlated the data with clinical findings.**

Experimental Design: **Gene promoter methylation was analyzed in 101 prostate cancer samples. In addition, we analyzed 32 nonmalignant prostate tissue samples, which included 25 with benign disease, benign prostatic hypertrophy, or prostatitis, and 7 normal tissues adjacent to cancer. The methylation status of 10 genes was determined. The methylation index (MI) was calculated as a reflection of the methylated fraction of the genes examined.**

Results: **Methylation percentages of the genes tested in prostate cancers were: *RAR*β, 53%; *RASSF1A*, 53%; *GSTP1*, 36%; *CDH13*, 31%; *APC*, 27%; *CDH1*, 27%; *FHIT*, 15%; $p16^{INK4A}$, 3%; *DAPK*, 1%; and *MGMT*, 0%. Methylation percentages in nonmalignant tissues were much lower. For clinicopathological correlations, we divided the cancer cases into low (6 or less) or high (7 or more) Gleason score (GS) groups, and into low (8 ng/ml or less) or high (greater than 8 ng/ml) preoperative serum prostate-specific antigen (PSA) groups. Methylation of *RASSF1A*, *GSTP1*, *RAR*β, and *CDH13* genes was significantly more frequent in the high GS group than in the low GS group. Methylation of *RASSF1A*, *CDH1*, and *GSTP1* genes was significantly more frequent in the high PSA group than in the low PSA group. The median MIs were significantly higher in the high GS and the high PSA groups. According to the Spearman rank-correlation test, there was significant correlation between MI and GS (coefficient = 0.43, $P < 0.0001$) and the preoperative serum PSA (coefficient = 0.37, $P = 0.0003$).**

Conclusions: **Our results indicate that the methylation profile of prostate cancers correlates with clinicopathological features of poor prognosis.**

INTRODUCTION

Prostate cancer is the most common malignancy and the second leading cause of cancer mortality among men in the United States (1). Once the tumor has metastasized, the long-term prognosis is poor because no curative therapy is available (2). Cancer development and metastasis are multistep processes that, among others, involve the inactivation of tumor suppressor genes. When the normal expression levels of these growth-inhibitory proteins are suppressed, uncontrolled cell cycling and growth can result. Identifying such specific molecular changes may contribute to prediction of the clinical state and aggressiveness of newly diagnosed prostate cancers.

DNA is methylated only at cytosines located 5′ to guanosine in the CpG dinucleotide, and DNA methylation of CpG sites in the promoter regions of genes is a frequent acquired epigenetic event in the pathogenesis of many human cancers (3, 4). This modification has important regulatory effects causing loss of gene expression, when there is involvement of CpG-rich areas known as CpG islands. Promoter region DNA methylation is acquired during tumorigenesis but is not normally present in nontumor tissue. DNA methylation may provide an alternate pathway to gene deletion or mutation for the loss of tumor suppressor gene function. Methylated DNA markers also represent a promising avenue for monitoring the onset and progression of cancer. Aberrant promoter methylation has been described for several genes in various malignancies, and the spectrum of genes involved suggests that specific tumors may have their own distinct pattern of methylation (4, 5).

For these reasons, we examined 10 genes the expression of which is frequently silenced by aberrant methylation in other cancers to develop a methylation profile for prostate cancer. We used the MSP[3] method (6), analyzed the frequency and extent of methylation (by determining the MI) for the 10 selected genes in prostate cancer, and correlated our findings with clinicopatho-

Received 9/4/01; revised 11/21/01; accepted 11/21/01.
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
[1] This research was supported by Grant U01CA84971 from the Early Detection Research Network, National Cancer Institute, and awards from the Nasher Family Cancer Program and the Amon Carter Foundation.
[2] To whom requests for reprints should be addressed, at Hamon Center for Therapeutic Oncology Research, University of Texas Southwestern Medical Center, 6000 Harry Hines Boulevard, Dallas, TX 75390-8593. Phone: (214) 648-4921; Fax: (214) 648-4940; E-mail: adi.gazdar@utsouthwestern.edu.

[3] The abbreviations used are: MSP, methylation-specific PCR; MI, methylation index; GS, Gleason score; PSA, prostate-specific antigen; FHIT, fragile histidine triad; RASSF1A, RAS association domain family protein 1A; RARβ, retinoic acid receptor β; APC, adenomatous polyposis coli; DAPK, death-associated protein kinase; MGMT, O^6-methylguanine-DNA-methyltransferase; GSTP1, glutathione *S*-transferase P1; CDH1, E-cadherin; CDH13, H-cadherin.

Table 1 Clinical characteristics of cancer patients

Characteristic	*n*
Total number	101
Median age (range)	63 (43–81) yrs
Race	
White	69
Black	15
Hispanic	5
Other	1
Unknown	11
Stage	
I	12
II	14
III	25
IV	9
Unknown	41
Gleason score	
4–5	22
6	14
7	25
8	20
9–10	20
Preoperative serum PSA	
<4	9
4–8	39
8.1–12	17
>12	28

Clinical staging of prostate cancer could only be performed on patients undergoing radical prostatectomy.

logical features known to be important for prognosis of prostate cancer patients.

MATERIALS AND METHODS

Clinical Samples. All prostate specimens were collected from 101 patients with prostate cancer, and adjacent nonmalignant tissue was available from 7 of these patients. In addition, we obtained nonmalignant tissues from 25 patients with benign prostatic hypertrophy or prostatitis. Patient operations were performed at Parkland Memorial or Zale-Lipshy University Hospitals, Dallas, TX, between 1994 and 2000, after Institutional Review Board-approved, signed consent was obtained. The patients underwent radical prostatectomy or transurethral resection. The tissue was maintained frozen in our urology tissue bank. The histological grading was according to the GS (7), and the stage of the disease was by the clinical TNM classification of the American Joint Committee on Cancer (8). The clinical characteristics of cancer patients are detailed in Table 1.

DNA Extraction and MSP. Genomic DNA was isolated from frozen tissue by digestion with 100 μg/ml proteinase K followed by standard phenol:chloroform (1:1) extraction and ethanol precipitation.

For MSP assays, DNA was treated with sodium bisulfite as described previously (6). Briefly, 1 μg of genomic DNA was denatured by incubation with 0.2 M NaOH for 10 min at 37°C. Aliquots of 10 mM hydroquinone (30 μl; Sigma Chemical Co., St. Louis, MO) and 3 M sodium bisulfite (pH 5.0, 520 μl; Sigma Chemical Co.) were added, and the solution (final volume 615.5 μl) was incubated at 50°C for 16 h. Treated DNA was purified by use of Wizard DNA Purification System (Promega Corp., Madison, WI), desulfonated with 0.3 M NaOH, precipitated with ethanol, and resuspended in water. Modified DNA was stored at −70°C until used. Gene location and primer sequences of all genes for both the methylated and the unmethylated forms, annealing temperatures, cycle number, and reference for methodology are summarized in Table 2. Two sets of primers were used to amplify each region of interest: one pair recognized a sequence in which CpG sites are unmethylated (bisulfite modified to UpG), and the other recognized a sequence in which CpG sites are methylated (unmodified by bisulfite treatment). Negative control samples without DNA were included for each set of PCR. PCR products were analyzed on 2% agarose gels containing ethidium bromide.

The precise conditions for MSP were selected so as to (*a*) distinguish between tumors and control tissues (buccal smears, peripheral blood cells) from healthy individuals, and (*b*) correlate methylation and gene silencing in cell lines. These conditions select for specificity over sensitivity. Under these conditions, no methylation of any of the genes tested was detected in 10 buccal smears and 10 peripheral blood sample DNAs from healthy volunteers. Specifically, we raised the annealing temperature for *GSTP1* from 59°C (9) to 64°C, because at 59°C some control buccal smears gave a MSP-positive band for *GSTP1*, whereas none were seen at 64°C. Because of the importance of *GSTP1* methylation in prostate cancer pathogenesis, we reconfirmed that methylation correlated 100% with gene expression in eight cell lines.

Data Analysis. A comparison of the proportion was done using the χ^2 test or Fisher's exact test. To compare the overall extent of methylation for the panel of genes examined, we calculated the MI for each case, as follows, and then determined the mean for the different groups.

$$\text{Methylation Index (MI)} = \frac{\text{Total no. of genes methylated}}{\text{Total no. of genes analyzed}}$$

Statistical analysis of MI between two variables was performed using the Mann-Whitney nonparametric *U* test. To examine the correlation between two variables, we used the Spearman rank-correlation test. A *P* less than 0.05 was defined as being statistically significant. All data were analyzed with the use of Survival Tools for StatView (Abacus Concepts Inc., Berkeley, CA).

RESULTS

Frequency of Methylation in Prostatic Tissues. The frequencies of methylation of the 10 genes in 101 prostate cancers and 32 nonmalignant prostate tissues (7 normal prostate tissues and 25 prostate gland tissues with benign disease) are detailed in Table 3. Methylation percentages in prostate cancers were: *RAR*β, 53%; *RASSF1A*, 53%; *GSTP1*, 36%; *CDH13*, 31%; *APC*, 27%; *CDH1*, 27%; *FHIT*, 15%; $p16^{INK4A}$, 3%; *DAPK*, 1%; and *MGMT*, 0%. The percentages of methylation in nonmalignant tissues were similar for *CDH1* and lower for the other genes. These differences were significant for *RAR*β, *RASSF1A*, *GSTP1*, *APC*, and *FHIT*. There was no methylation of *FHIT*, $p16^{INK4A}$, or *DAPK* in nonmalignant tissues. Most nonmalignant tissue (18 of 32, 56%) lacked methylation of any gene, and none had methylation of four or more genes. By

Table 2 Summary data of genes tested

Gene	Gene location	Forward primer	Reverse primer	Temperature (°C)	Cycle no.	Reference no.
FHIT	3p14	M: 5′-TTGGGGCGCGGTTTGGTTTTACGC-3′[a]	M: 5′-CGTAAACGACGCCGACCCCACTA-3′	71–64	40	33
		U: 5′-TTGGGGTGTGGGTTTGGTTTTATG-3′	U: 5′-CATAAACAACACCAACCCCACTA-3′	71–64	40	
RASSF1A	3p21	M: 5′-GGGTTTTGCGAGAGCGCG-3′	M: 5′-GCTAACAAACGCGAACCG-3′	64	40	17
		U: 5′-GGTTTTGTGAGAGTGTGTTTAG-3′	U: 5′-CACTAACAAACACAAACCAAAC-3′	59	40	
*RAR*β	3p24	M: 5′-TCGAGAACGCGAGCGATTCG-3′	M: 5′-GACCAATCCAACCGAAACGA-3′	62	35	16
		U: 5′-TTGAGAAATGTGAGTGATTTGA-3′	U: 5′-AACCAATCCAACCAAAACAA-3′	62	35	
APC	5q21	M: 5′-TATTGCGGAGTGCGGTC-3′	M: 5′-TCGACGAACTCCCGACGA-3′	64	35	34
		U: 5′-GTGTTTTATTGTGAGTGTGGGTT-3′	U: 5′-CCAATCAACAAACTCCCAACAA-3′	62	35	
p16^{INK4A}	9p21	M: 5′-TTATTAGAGGGTGGGGCGGATCGC-3′	M: 5′-GACCCCGAACCGCGACCGTAA-3′	65	35	6
		U: 5′-TTATTAGAGGGTGGGGTGGATTGT-3′	U: 5′-CAACCCCAAACCACACAACCATAA-3′	60	35	
DAPK	9q34	M: 5′-GGATAGTCGGATCGAGTTAACGTC-3′	M: 5′-CCCTCCCAAACGCCGA-3′	64	35	35
		U: 5′-GGAGGATAGTTGGATTGAGTTAATGTT-3′	U: 5′-CAAATCCCTCCCAAACACCAA-3′	64	35	
MGMT	10q26	M: 5′-TTTCGACGTTCGTAGGTTTTCGC-3′	M: 5′-GCACTCTTCCGAAAACGAAACG-3′	66	35	36
		U: 5′-TTTGTGTTTTGATGTTTGTAGGTTTTTGT-3′	U: 5′-AACTCCACACTCTTCCAAAAACAAAACA-3′	66	35	
GSTP1	11q13	M: 5′-TTCGGGGTGTAGCGGTCGTC-3′	M: 5′-GCCCCAATACTAAATCACGACG-3′	64	37	9
		U: 5′-GATGTTTGGGGTGTAGTGGTTGTT-3′	U: 5′-CCACCCCAATACTAAATCACAACA-3′	64	37	
CDH1	16q22	M: 5′-GGTGAATTTTTAGTTAATTAGCGGTAC-3′	M: 5′-CATAACTAACCGAAAACGCCG-3′	57	37	37
		U: 5′-GGTAGGTGAATTTTTAGTTAATTAGTGGTA-3′	U: 5′-ACCCATAACTAACCAAAAACACCA-3′	57	37	
CDH13	16q24	M: 5′-TCGCGGGGTTCGTTTTTCGC-3′	M: 5′-GACGTTTTCATTCATACACGCG-3′	64	37	22
		U: 5′-CGCGGGGCTCGCTCCTCGC-3′	U: 5′-GGCGTTTTCATTCATGCACGCG-3′	64	37	

[a] M, methylated-specific primers; U, unmethylated-specific primers.

Table 3 Frequency of aberrant methylation in prostatic tissues

Gene	Cancers (%) (n = 101)	Nonmalignant (%) (n = 32)	P
*RAR*β	54 (53)	1 (3)	<0.0001
RASSF1A	54 (53)	5 (16)	0.0002
GSTP1	36 (36)	1 (3)	0.0002
APC	27 (27)	2 (6)	0.01
FHIT	15 (15)	0	0.02
CDH13	31 (31)	5 (16)	0.1
CDH1	27 (27)	8 (25)	>0.5
p16^{INK4A}	3 (3)	0	>0.5
DAPK	1 (1)	0	>0.5
MGMT	0	0	>0.5

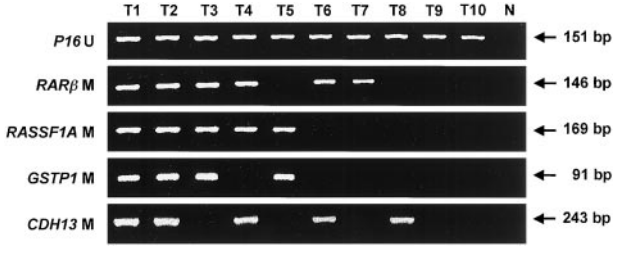

Fig. 1 Representative examples of MSP analyses of the methylated form (*M*) of four genes frequently methylated in prostate cancers, *RAR*β, *RASSF1A*, *GSTP1*, and *CDH13*. Amplification of the unmethylated form of *p16*^{INK4A} (*p16*U) was used as a control for DNA integrity. *T*, tumor samples 1 through 10; *N*, negative control.

contrast, most cancers (81 of 101, 80%) had methylation of one or more genes, and 32 (32%) had methylation of four to seven genes. Fig. 1 illustrates representative examples of the methylation patterns in tumors of the four most frequently methylated genes. The unmethylated form of *p16*^{INK4A}, run as a control for DNA integrity, was present in all samples.

Methylation and Clinicopathological Correlations. The correlation of methylation frequencies and the MI are shown in Fig. 2; and these are related to GS (Fig. 2*A*), the preoperative serum PSA (Fig. 2*B*), and tumor stage (Fig. 2*C*), three known clinicopathological factors for prostate cancer patient survival. Prostatic tumors with GS values of 5–6 have a significantly better clinical course than those with values of 7 or greater (10). For this reason, we divided our tumors into those with values of 6 or lower (low GS group) and those with values of 7 or greater (high GS group). The median value of preoperative serum PSA in our patients was 7.5 ng/ml. To obtain approximately equal numbers in each group, we divided our patients into a "low PSA" group (8 ng/ml or less), and a "high PSA" group (greater than 8 ng/ml). The median values of PSA in the low and high PSA groups were 5.6 and 14.2 ng/ml, respectively. Staging information was available for 60 of the patients. Because of the relatively small numbers in each of the four grades, we pooled patients into low stage (stages I and II) or high stage (stages III and IV) groups.

The median MIs of the high GS, the high PSA, and the high stage groups were significantly greater than those of corresponding low groups. All three high value groups had significantly greater methylation frequencies of the *GSTP1* gene, and

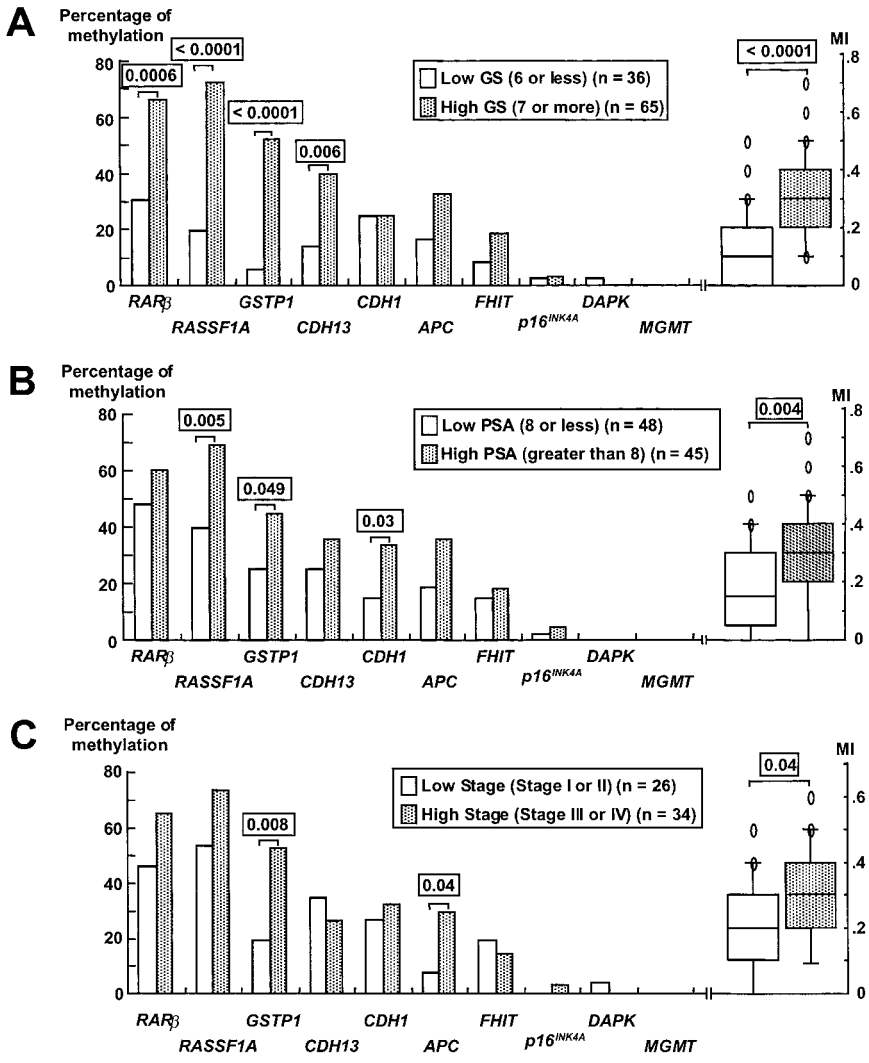

Fig. 2 Correlation of methylation findings with the low and high groups for GS (*A*), preoperative serum PSA (*B*), and tumor stage (*C*). On the *right side* of each *panel* are illustrated the corresponding MIs.

the high GS and PSA groups had a significantly greater frequency of methylation of *RASSF1A*, whereas the high GS group also had a significantly higher *RAR*β and *CDH13* methylation frequency. According to the Spearman rank-correlation test, there was a significant correlation between MI and GS (coefficient = 0.43, $n = 101$, $P < 0.0001$), and between the MI and the preoperative serum PSA (coefficient = 0.37, $n = 93$, $P = 0.0003$).

Survival data were available for 44 patients with a median follow-up period of 27 months. Although methylation status did not correlate with survival, no deaths were noted until 48 months after surgery. Thus, a lengthy follow-up will be required to determine whether methylation is a predictive factor for survival.

DISCUSSION

Previous studies have described the importance of DNA methylation in human cancers and have focused on regions of the genome that may have functional significance resulting from the extinction of gene activity. Whereas most individual cancers have several, perhaps hundreds, of methylated genes (4), the methylation profiles of individual tumor types are characteristic (4, 5, 11).

Of the 10 genes studied, we found 6 genes (*RAR*β, *RASSF1A*, *GSTP1*, *CDH13*, *APC*, and *CDH1*) that were selectively methylated in prostate cancer tissues at frequencies greater than 25%. One gene (*FHIT*) was occasionally methylated, whereas three genes ($p16^{INK4A}$, *DAPK*, and *MGMT)* were seldom if ever methylated. Of particular interest, the MI and the frequencies of methylation of three or four genes were significantly higher in tumors of patients with increased risk of death, namely high GS, high preoperative PSA, and high stage. Other studies have demonstrated that the preoperative serum PSA reflects both tumor grade (12) and tumor volume (13).

Several reports indicate that *GSTP1* methylation is present in prostate cancers at very high frequencies (9, 14). Our finding of a methylation rate of 36% in prostate tumors is lower than most other reports and may reflect the use of a higher annealing temperature in our MSP assay, which provides for specificity over sensitivity. Using our conditions, several control tissues

from healthy individuals (data not shown) and all but one sample of nonmalignant prostate tissue were negative for methylation. In addition, there was a complete concordance between methylation and expression of the gene in eight tumor cell lines. Other reports have failed to find an association between *GSTP1* methylation and markers of increased risk. In the high risk groups, the methylation frequencies of *GSTP1* were between 44% and 53%. Our findings that frequent methylation of *GSTP1* was correlated with markers of increased risk suggest that our stringent conditions may yield more clinically useful information.

Our finding of a low incidence of $p16^{INK4A}$ methylation in prostate cancers is consistent with published data (15). There is little published information about the other genes we studied. Retinoic acid plays an important role in lung development and differentiation, acting primarily via nuclear receptors encoded by *RAR*β gene. Because the isoforms *RAR*β*2* and *RAR*β*4* are frequently methylated in lung and breast cancers (16), we investigated methylation of their promoter, P2, and found frequent methylation in the prostate cancers (53%). Our gene panel included *RASSF1*, a recently identified tumor suppressor gene. There are two major *RASSF1* gene products, *RASSF1A* and *RASSF1C*. Selective promoter methylation of the *RASSF1A* promoter, but not of *RASSF1C*, is frequent in many cancers including lung, breast, nasopharyngeal, ovarian, and renal carcinomas and mesotheliomas (17–21). The *RASSF1A* promoter was methylated frequently in prostate cancers (53%), and methylation was associated with high GS and preoperative serum PSA. These findings indicate a potential role of *RASSF1A* in the pathogenesis and spread of prostate cancers.

Inactivation of *CDH13* resulting from aberrant methylation and other mechanisms is frequent in some types of cancer (22, 23). We found methylation of *CDH13* in 31% of prostate cancers and 20% of benign diseases of prostate. Our findings suggest that methylation of *CDH13* is an early event during the pathogenesis of prostate cancer. Inactivation of the *APC* gene is frequent in colorectal and other gastrointestinal carcinomas, usually by truncating mutations (24). An alternative method of inactivation of the gene in some gastrointestinal cancers is by promoter methylation (25). Recently we reported that selective methylation and silencing of the *APC* 1A promoter and its specific products were frequent in lung and breast cancers (26). We found methylation of *APC* 1A in 27% of prostate cancers. It has been demonstrated that decreased expression of *CDH1* is associated with high grade, advanced stage prostate tumors (27), and methylation of a subset of prostate cancer cell lines has been described (28). We found methylation of *CDH1* in 27% of prostate cancers, indicating that aberrant methylation may be the major mechanism of silencing of this gene. Guo *et al.* (29) demonstrated down-regulation of FHIT protein in more than half of the primary prostate cancers examined by immunostaining. However, another study failed to identify the mechanism of inactivation (30). We found methylation of *FHIT* in 15% of prostate cancers, indicating that promoter methylation of the *FHIT* gene is one of the mechanisms involved in gene silencing.

Methylation of some genes (*CDH1*, *CDH13*, and *RASSF1A*) were occasionally methylated in nonmalignant prostatic tissues (benign prostatic hypertrophy and prostatitis). Of interest, *GSTP1* was methylated in only 3% of these tissues. Methylation has been described in various nonmalignant tissues as an aging change or following chronic inflammation (31, 32).

We have identified several new genes methylated at relatively high frequencies in prostate cancer. Our results indicate that the methylation profile of prostate cancers correlates with clinicopathological features of poor prognosis. Although our findings need to be extended to a larger series, our results suggest that the methylation profile of genes may be a potential new biomarker for determining the prognosis of prostate cancer patients.

REFERENCES

1. Greenlee, R. T., Murray, T., Bolden, S., and Wingo, P. A. Cancer statistics 2000. CA - Cancer J. Clin., *50:* 7–33, 2000.

2. Johansson, J. E., Holmberg, L., Johansson, S., Bergstrom, R., and Adami, H. O. Fifteen-year survival in prostate cancer. A prospective, population-based study in Sweden [see comments; published erratum in JAMA *278:* 206, 1997]. JAMA, *277:* 467–471, 1997.

3. Baylin, S. B., Herman, J. G., Graff, J. R., Vertino, P. M., and Issa, J. P. Alterations in DNA methylation: a fundamental aspect of neoplasia. Adv. Cancer Res., *72:* 141–196, 1998.

4. Costello, J. F., Fruhwald, M. C., Smiraglia, D. J., Rush, L. J., Robertson, G. P., Gao, X., Wright, F. A., Feramisco, J. D., Peltomaki, P., Lang, J. C., Schuller, D. E., Yu, L., Bloomfield, C. D., Caligiuri, M. A., Yates, A., Nishikawa, R., Su Huang, H., Petrelli, N. J., Zhang, X., O'Dorisio, M. S., Held, W. A., Cavenee, W. K., and Plass, C. Aberrant CpG-island methylation has non-random and tumour-type-specific patterns [see comments]. Nat. Genet., *24:* 132–138, 2000.

5. Esteller, M., Corn, P. G., Baylin, S. B., and Herman, J. G. A gene hypermethylation profile of human cancer. Cancer Res., *61:* 3225–3229, 2001.

6. Herman, J. G., Graff, J. R., Myohanen, S., Nelkin, B. D., and Baylin, S. B. Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. Proc. Natl. Acad. Sci. USA, *93:* 9821–9826, 1996.

7. Gleason, D. F., and Mellinger, G. T. Prediction of prognosis for prostatic adenocarcinoma by combined histological grading and clinical staging. J. Urol., *111:* 58–64, 1974.

8. Beahrs, O. H., Henson, D. E., and Hutter, R. V. P. American Joint Committee on Cancer: Manual for Staging Cancer, Ed. 4. Philadelphia: Lippincott, 1992.

9. Esteller, M., Corn, P. G., Urena, J. M., Gabrielson, E., Baylin, S. B., and Herman, J. G. Inactivation of glutathione S-transferase P1 gene by promoter hypermethylation in human neoplasia. Cancer Res., *58:* 4515–4518, 1998.

10. Tefilli, M. V., Gheiler, E. L., Tiguert, R., Sakr, W., Grignon, D. J., Banerjee, M., Pontes, J. E., and Wood, D. P., Jr. Should Gleason score 7 prostate cancer be considered a unique grade category? Urology, *53:* 372–377, 1999.

11. Zochbauer-Muller, S., Fong, K. M., Virmani, A. K., Geradts, J., Gazdar, A. F., and Minna, J. D. Aberrant promoter methylation of multiple genes in non-small cell lung cancers. Cancer Res., *61:* 249–255, 2001.

12. Aihara, M., Lebovitz, R. M., Wheeler, T. M., Kinner, B. M., Ohori, M., and Scardino, P. T. Prostate specific antigen and Gleason grade: an immunohistochemical study of prostate cancer. J. Urol., *151:* 1558–1564, 1994.

13. Stamey, T. A., McNeal, J. E., Yemoto, C. M., Sigal, B. M., and Johnstone, I. M. Biological determinants of cancer progression in men with prostate cancer. JAMA, *281:* 1395–1400, 1999.

14. Lee, W. H., Isaacs, W. B., Bova, G. S., and Nelson, W. G. CG island methylation changes near the *GSTP1* gene in prostatic carcinoma cells detected using the polymerase chain reaction: a new prostate cancer biomarker. Cancer Epidemiol. Biomark. Prev., *6:* 443–450, 1997.

15. Jarrard, D. F., Bova, G. S., Ewing, C. M., Pin, S. S., Nguyen, S. H., Baylin, S. B., Cairns, P., Sidransky, D., Herman, J. G., and Isaacs, W. B. Deletional, mutational, and methylation analyses of CDKN2 (p16/MTS1) in primary and metastatic prostate cancer. Genes Chromosomes Cancer, *19:* 90–96, 1997.

16. Virmani, A. K., Rathi, A., Zochbauer-Muller, S., Sacchi, N., Fukuyama, Y., Bryant, D., Maitra, A., Heda, S., Fong, K. M., Thunnissen, F., Minna, J. D., and Gazdar, A. F. Promoter methylation and silencing of the retinoic acid receptor-β gene in lung carcinomas. J. Natl. Cancer Inst., *92:* 1303–1307, 2000.

17. Burbee, D. G., Forgacs, E., Zochbauer-Muller, S., Shivakumar, L., Fong, K., Gao, B., Randle, D., Kondo, M., Virmani, A., Bader, S., Sekido, Y., Latif, F., Milchgrub, S., Toyooka, S., Gazdar, A. F., Lerman, M. I., Zabarovsky, E., White, M., and Minna, J. D. Epigenetic inactivation of RASSF1A in lung and breast cancers and malignant phenotype suppression. J. Natl. Cancer Inst., *93:* 691–699, 2001.

18. Dammann, R., Li, C., Yoon, J. H., Chin, P. L., Bates, S., and Pfeifer, G. P. Epigenetic inactivation of a RAS association domain family protein from the lung tumour suppressor locus 3p21.3. Nat Genet., *25:* 315–319, 2000.

19. Agathanggelou, A., Honorio, S., Macartney, D. P., Martinez, A., Dallol, A., Rader, J., Fullwood, P., Chauhan, A., Walker, R., Shaw, J. A., Hosoe, S., Lerman, M. I., Minna, J. D., Maher, E. R., and Latif, F. Methylation associated inactivation of RASSF1A from region 3p21.3 in lung, breast and ovarian tumours. Oncogene, *20:* 1509–1518, 2001.

20. Toyooka, S., Pass, H. I., Shivapurkar, N., Fukuyama, Y., Maruyama, R., Toyooka, K. O., Gilcrease, M., Farinas, A., Minna, J. D., and Gazdar, A. F. Aberrant methylation and simian virus 40 tag sequences in malignant mesothelioma. Cancer Res., *61:* 5727–5730, 2001.

21. Lo, K. W., Kwong, J., Hui, A. B., Chan, S. Y., To, K. F., Chan, A. S., Chow, L. S., Teo, P. M., Johnson, P. J., and Huang, D. P. High frequency of promoter hypermethylation of RASSF1A in nasopharyngeal carcinoma. Cancer Res., *61:* 3877–3881, 2001.

22. Sato, M., Mori, Y., Sakurada, A., Fujimura, S., and Horii, A. The H-cadherin (*CDH13*) gene is inactivated in human lung cancer [Published erratum in Hum Genet., *103:* 532, 1998]. Hum. Genet., *103:* 96–101, 1998.

23. Toyooka, K. O., Toyooka, S., Virmani, A. K., Sathyanarayana, U. G., Euhus, D. M., Gilcrease, M., Minna, J. D., and Gazdar, A. F. Loss of expression and aberrant methylation of the *CDH13* (H-cadherin) gene in breast and lung carcinomas. Cancer Res., *61:* 4556–4560, 2001.

24. Nagase, H., and Nakamura, Y. Mutations of the *APC* (adenomatous polyposis coli) gene. Hum. Mutat., *2:* 425–434, 1993.

25. Esteller, M., Sparks, A., Toyota, M., Sanchez-Cespedes, M., Capella, G., Peinado, M. A., Gonzalez, S., Tarafa, G., Sidransky, D., Meltzer, S. J., Baylin, S. B., and Herman, J. G. Analysis of adenomatous polyposis coli promoter hypermethylation in human cancer. Cancer Res., *60:* 4366–4371, 2000.

26. Virmani, A. K., Rathi, A., Sathyanarayana, U. G., Padar, A., Huang, C. X., Cunnigham, H. T., Farinas, A. J., Milchgrub, S., Euhus, D. M., Gilcrease, M., Herman, J., Minna, J. D., and Gazdar, A. F. Promoter methylation and inactivation of the adenomatous polyposis coli (*APC*) gene in breast and lung carcinomas. Clin. Cancer Res., *7:* 1998–2004, 2001.

27. De Marzo, A. M., Knudsen, B., Chan-Tack, K., and Epstein, J. I. E-cadherin expression as a marker of tumor aggressiveness in routinely processed radical prostatectomy specimens. Urology, *53:* 707–713, 1999.

28. Graff, J. R., Herman, J. G., Lapidus, R. G., Chopra, H., Xu, R., Jarrard, D. F., Isaacs, W. B., Pitha, P. M., Davidson, N. E., and Baylin, S. B. E-cadherin expression is silenced by DNA hypermethylation in human breast and prostate carcinomas. Cancer Res., *55:* 5195–5199, 1995.

29. Guo, Z., Johansson, S. L., Rhim, J. S., and Vishwanatha, J. K. Fragile histidine triad gene expression in primary prostate cancer and in an in vitro model. Prostate, *43:* 101–110, 2000.

30. Latil, A., Bieche, I., Fournier, G., Cussenot, O., Pesche, S., and Lidereau, R. Molecular analysis of the *FHIT* gene in human prostate cancer. Oncogene, *16:* 1863–1868, 1998.

31. Issa, J. P. CpG-island methylation in aging and cancer. Curr. Top. Microbiol. Immunol., *249:* 101–118, 2000.

32. Kaneto, H., Sasaki, S., Yamamoto, H., Itoh, F., Toyota, M., Suzuki, H., Ozeki, I., Iwata, N., Ohmura, T., Satoh, T., Karino, Y., Toyota, J., Satoh, M., Endo, T., Omata, M., and Imai, K. Detection of hypermethylation of the *p16(INK4A)* gene promoter in chronic hepatitis and cirrhosis associated with hepatitis B or C virus. Gut, *48:* 372–377, 2001.

33. Zochbauer-Muller, S., Fong, K. M., Maitra, A., Lam, S., Geradts, J., Ashfaq, R., Virmani, A. K., Milchgrub, S., Gazdar, A. F., and Minna, J. D. 5′CpG island methylation of the *FHIT* gene is correlated with loss of gene expression in lung and breast cancer. Cancer Res., *61:* 3581–3585, 2001.

34. Tsuchiya, T., Tamura, G., Sato, K., Endoh, Y., Sakata, K., Jin, Z., Motoyama, T., Usuba, O., Kimura, W., Nishizuka, S., Wilson, K. T., James, S. P., Yin, J., Fleisher, A. S., Zou, T., Silverberg, S. G., Kong, D., and Meltzer, S. J. Distinct methylation patterns of two *APC* gene promoters in normal and cancerous gastric epithelia. Oncogene, *19:* 3642–3646, 2000.

35. Esteller, M., Sanchez-Cespedes, M., Rosell, R., Sidransky, D., Baylin, S. B., and Herman, J. G. Detection of aberrant promoter hypermethylation of tumor suppressor genes in serum DNA from non-small cell lung cancer patients [published erratum in Cancer Res., *59:* 3853, 1999]. Cancer Res., *59:* 67–70, 1999.

36. Esteller, M., Hamilton, S. R., Burger, P. C., Baylin, S. B., and Herman, J. G. Inactivation of the DNA repair gene *O*-6-methylguanine-DNA methyltransferase by promoter hypermethylation is a common event in primary human neoplasia. Cancer Res., *59:* 793–797, 1999.

37. Graff, J. R., Herman, J. G., Myohanen, S., Baylin, S. B., and Vertino, P. M. Mapping patterns of CpG island methylation in normal and neoplastic cells implicates both upstream and downstream regions in de novo methylation. J. Biol. Chem., *272:* 22322–22329, 1997.

Clinical Cancer Research

Aberrant Promoter Methylation Profile of Prostate Cancers and Its Relationship to Clinicopathological Features

Riichiroh Maruyama, Shinichi Toyooka, Kiyomi O. Toyooka, et al.

Clin Cancer Res 2002;8:514-519.