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DNA Hypermethylation Status of Multiple Genes in Prostate Adenocarcinomas

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Multiple genetic mutations and epigenetic methylation are believed to be involved in prostate carcinogenesis, but it is not known whether these events are independent or correlated in some fashion. We therefore studied 32 prostate adenocarcinomas not only for deletions and/or mutations of multiple suspect genes, but also for aberrant DNA methylation using methylation-specific PCR (MSP). Of those genes examined, *p16^{INK4a}*, *O^6-MGMT*, and *GST-P* were found to be the most frequently methylated (66%, 25% and 75% of cases, respectively), while methylations of *p14^{ARF}*, *RB1*, *p21^{Waf1}*, and *p27^{Kip1}* were far less common (3%, 6%, 6% and 6% of cases, respectively). Methylation of *O^6-MGMT* and *GST-P* genes was defective in about 19% of the cases and there were occasional simultaneous deletions and methylations of *p14^{ARF}* and *p16^{INK4a}* genes (13% and 3% of cases, respectively). In *p16^{INK4a}*, methylation occurred in the promoter region in 9% of samples and in exon 2 in 66% of tumors. Hypermethylation of *O^6-MGMT* with concurrent *p53* and *ras* gene mutations were found in 6% and 13% of specimens, respectively; among those tumors with high Gleason scores were 2 carcinomas showing hypermethylated *O^6-MGMT* with G-to-A transitions in K-*ras*. Our results demonstrate that multiple genes of a subset common in prostate carcinomas are methylated and not infrequently show concurrent deletions. Further, there is a suggestion that specific combinations of hypermethylation and mutation correlate to tumor malignancy.

Key words: Prostate carcinoma — Methylation — Mutation — Gene

Prostate carcinoma is one of the most common malignancies worldwide, particularly in Western countries. The development and progression of prostate cancer appear to result from an accumulation of sequential genetic events, but there is little real evidence to support single genetic alterations as the sole cause of carcinogenesis in the prostate. Multiple oncogenes and tumor suppressor genes have been investigated recently by advanced molecular methods; however, it is difficult to translate this molecular knowledge into widely applicable diagnostic and prognostic criteria in the management and treatment of the disease.

Many studies on prostate cancer progression imply as yet unknown epigenetic mechanisms.[1] Apart from specific genetic mutations, recent studies have demonstrated silencing of tumor suppressor genes by promoter hypermethylation as a common feature in human tumors. Hypermethylation in the promoter regions of *p14^{ARF}*, *p15^{INK4b}*, *p16^{INK4a}*, *GST-P*, *E-cadherin* and *VHL* genes have been well described.[2–4] The identification of genes susceptible to hypermethylation may provide insight into cancers driven by this particular pathway. Therefore, the accumulation of genetic and epigenetic alterations must be responsible for prostate cancer development and for subsequent progression.

On the basis of these previous observations of *ras*, *p53* and *RB1* gene mutations, we examined 32 prostate carcinomas using methylation-specific PCR (MSP),[2] differential PCR[5] and PCR-SSCP analyses to look for hypermethylation and mutations in multiple genes implicated in prostate cancers, such as *p14^{ARF}*, *p16^{INK4a}*, *RB1*, *p21^{Waf1}*, *p27^{Kip1}*, *PTEN*, *p73*, *p53*, *O^6-MGMT*, *ras* and *GST-P*. Our results indicate that carcinomas of the prostate develop through distinct epigenetic and/or genetic pathways having different molecular profiles.

MATERIALS AND METHODS

Tumor samples and DNA extraction Thirty-two samples of prostate carcinomas and the 8 corresponding normal tissues were obtained from total prostatectomies and transurethral resections; Tumor samples were fixed in 10% buffered formalin, embedded in paraffin, cut at 4 μm, and stained with hematoxylin and eosin for pathological evaluation. Tumors were graded according to Gleason's histological criteria.[6] The equivalent portions of tissues were frozen at −70°C for later DNA extraction as described previously.[7]

Methylation-specific PCR DNA methylation patterns in the CpG islands of the *p14^{ARF}*, *p16^{INK4a}*, *RB1*, *p21^{Waf1}*, *p27^{Kip1}*, *PTEN*, *p73*, *O^6-MGMT* and *GST-P* were determined by MSP.[2] MSP distinguishes unmethylated from methylated alleles based on sequence changes produced after bisulfite treatment of DNA, which converts unmethylated (but not methylated) cytosine to uracil, and subsequent PCR using primers designed for either methylated or

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unmethylated DNA. MSP can detect 1 methylated allele among 1000 unmethylated ones.[2] Sodium bisulfite modification was performed using the "CpGenome" DNA Modification Kit (Intergen, Oxford, UK) according to the manufacturer's protocol with additional minor modifications.[8, 9] Briefly, DNA was denatured by NaOH (final conc. 0.2 *M*) for 15 min at 37°C. Sodium bisulfite solution at pH 5, freshly prepared, was added (550 μl), and incubated at 50°C for 20 h. The modified DNA was treated with NaOH (final conc. 0.3 *M*) for 5 min at room temperature, followed by ethanol precipitation, and was resuspended. Control methylated (Intergen) and unmethylated DNA from blood of normal volunteers were treated with bisulfite as mentioned above.

The primer sequences for methylated and unmethylated PCR and the MSP conditions have been previously reported[2, 10–17] for *p14^{ARF}*, *p16^{INK4a}*, *RB1*, *p21^{Waf1}*, *p27^{Kip1}*, *p73*, *O^6-MGMT* and *GST-P*. The primer sequences for methylated and unmethylated PCR of *PTEN* were as follows: 5′-TTT TCG TTC GGC GCG GTT TCG-3′ (sense) and 5′-GCC GCG CCG AAA ACC CGA ACG-3′ (antisense) for the methylated reaction; 5′-TTG TTT GGT GTG GTT TTG TTT GTT T-3′ (sense) and 5′-ACC ACC ACA CCA AAA ACC CAA ACA-3′ (antisense) for the unmethylated reaction. The annealing temperature for both *PTEN* methylated and unmethylated reactions was 64°C, while that for both *p73* methylated and unmethylated reactions was 60°C. Amplified products were electrophoresed on 2% agarose gels and visualized by ethidium bromide staining.

Differential PCR for *p14^{ARF}* and *p16^{INK4a}* deletions To assess homozygous deletions, we carried out differential PCR.[5] Duplex PCR amplification was performed to generate a 149 bp fragment covering exon 1β of the *p14^{ARF}* gene and a 204-bp exon fragment of *p16^{INK4a}* 1α, together with a 160 bp fragment of the *GAPDH* gene and a 187 bp fragment of *β-actin* as a reference, respectively. The *GAPDH* and *β-actin* genes were used as internal controls, mainly to demonstrate that the template was intact and also to enable us to control for contamination of samples by normal cells. Both sets of primers from the control locus and potentially deleted locus were included in the PCR reaction mixture. DNA was amplified with 30 and 29 cycles of PCR for the *p14^{ARF}* and *p16^{INK4a}* genes, respectively. The primer sequences and detailed PCR conditions were previously described.[9] The PCR product was analyzed on an 8% acrylamide gel. Gels were photographed using a DC290 Zoom Digital Camera (Eastman Kodak, Rochester, NY) and densitometry of the PCR fragments was performed using Kodak Digital Science ID Image Analysis Software (Ver. 3.5.2, Kodak, NY). Samples presenting <20% of the control signal were considered homozygously deleted.[9, 18] To assess the sensitivity of differential PCR for *p16^{INK4a}* and *p14^{ARF}* homozygous deletion, we carried out a titration experiment with various ratios of normal DNA and DNA from A172 glioma cells with homozygous co-deletion of the *p16^{INK4a}* and *p14^{ARF}* genes.[19] The chosen threshold value of 0.2 allows the detection of homozygous deletion in the presence of approximately 30% normal DNA for *p16^{INK4a}* and 25% for *p14^{ARF}*.[9]

***ras* and *p53* mutations** Exons 4–9 of *p53* and of the K-, N-, and H-*ras* genes were amplified by PCR and screened for mutations by SSCP analysis as described previously.[20, 21]

Statistical differences at a given locus were analyzed by the Mann-Whitney *U* test, a *P* value less than 0.05 being considered significant.

RESULTS

***p14^{ARF}* and *p16^{INK4a}* alterations and *p53* status** Hypermethylation of *p16^{INK4a}* exon 2 was found in 21 cases (~66%), within which 3 samples (nos 4, 5, and 10) demonstrated promoter methylation as well as methylation of exon 2 (Table I). Methylated and unmethylated DNAs showed the expected fragment size of 122 bp and 132 bp for *p14^{ARF}*, and 150 bp and 151 bp for *p16^{INK4a}*.

Simultaneous homozygous deletion of the *p14^{ARF}* and *p16^{INK4a}* gene was detected by differential PCR in 4 of 32 (13%) samples (Table I and Fig. 1). Hypermethylation of both the *p14^{ARF}* and *p16^{INK4a}* promoters was detected in only 1 case, specimen no. 10 (Table I and Fig. 2A). A total of 8 tumors (25%) showed either *p16^{INK4a}* deletions, or promoter methylation or exon mutations; an additional specimen, no. 4, demonstrated concurrent promoter methylation and an intron mutation in *p16^{INK4a}*. As was previously reported,[20] there appears to be a mutually exclusive correlation between *p14^{ARF}* and *p53* status, i.e., cases having *p14^{ARF}* mutations showed no *p53* mutations. One tumor (case 10), however, did show *p14^{ARF}* promoter methylation and a G→C transversion in exon 4 of *p53*.

***RB1*, *p21^{Waf1}*, *p27^{Kip1}*, *PTEN* and *p73* methylation** Methylated and unmethylated control DNAs showed the expected fragment size of 163 bp for *RB1*, 108 bp and 111 bp for *p21^{Waf1}* and 195 bp and 212 bp for *p27^{Kip1}*. *RB1*, *p21^{Waf1}* and *p27^{Kip1}* promoter hypermethylation was detected in 2 cases each. Independent *RB1* mutations or loss of heterozygosity at the *RB1* locus have been reported[22] and were also found in this study (sample nos 2, 6, 9, 14, 17, 21, and 27). Methylation of the *RB1* promoter coupled to an intronic mutation occurred in a single sample (no. 26). In a single tumor (case no. 11), hypermethylation of both *p21^{Waf1}* and *p27^{Kip1}* was detected (Fig. 2B); two separate carcinomas were methylated independently (Table I). Methylation of *PTEN* and *p73* was not detected (Table I and Fig. 3).

***O^6*-MGMT and *GST-P* methylation and *ras* mutations** *O^6-MGMT* methylation was detected in 7 (~22%)

Table I. Alterations of *INK4A/ARF* Locus and Methylation Profile of Multiple Genes in Prostate Carcinomas

Case no.	Gleason score	*p14ARF*		*p16INK4a*				*RB1*			*p21Waf1*	*p27Kip1*	*PTEN*	*p53*	*p73*	*O6-MGMT*	*ras*	*GST-P*
		Prom	E 1β	Prom	E 2	E 1α	Mut	Prom	Mut	LOH				Mut				
1	9	—	—	—	Methyl	—	—	—	—	—	—	—	—	—	—	—	—	Methyl
2	8	—	Del	—	Methyl	Del	—	—	—	LOH	—	—	—	—	—	Methyl	K-12/G→A	Methyl
3	5	—	Del	—	Methyl	Del	—	—	—	—	—	—	—	—	—	—	—	Methyl
4	7	—	—	Methyl	Methyl	—	I 2	—	—	—	—	—	—	—	—	—	—	Methyl
5	6	—	—	Methyl	Methyl	—	—	—	—	—	—	—	—	—	—	—	—	—
6	6	—	—	—	—	—	E 1	—	I 6	—	—	—	—	—	—	—	—	Methyl
7	9	—	—	—	—	—	—	—	—	—	—	—	—	—	—	Methyl	K-12/G→A	Methyl
8	9	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	H-61	—
9	7	—	—	—	—	—	E 2	—	—	LOH	—	—	—	—	—	—	—	Methyl
10	6	Methyl	—	Methyl	Methyl	—	—	—	—	—	—	—	—	E 4/G→C	—	—	—	Methyl
11	6	—	—	—	—	—	—	—	—	—	Methyl	Methyl	—	—	—	—	—	Methyl
12	7	—	—	—	Methyl	—	—	—	—	—	—	—	—	—	—	—	—	—
13	9	—	—	—	Methyl	—	—	—	—	—	—	—	—	—	—	—	—	Methyl
14	7	—	—	—	—	—	—	—	—	LOH	—	—	—	—	—	—	—	Methyl
15	5	—	—	—	Methyl	—	—	—	—	—	—	—	—	E 7/A→T	—	Methyl	—	—
16	9		—		—	—	—	—	—	—	—	—	—	—	—	—	—	Methyl
17	3	—		—	Methyl	—	—	—	E 17	—	—	—	—	—	—	—	—	Methyl
18	7	—	—	—	Methyl	—	—	—	—	—	—	—	—	E 5/A→T	—	—	H-61	Methyl
19	9	—	Del	—	—	Del	—	—	—	—	—	—	—	—	—		—	Methyl
20	5	—	—	—	—	—	—	—	—	—	—	—	—	—	—		—	Methyl
21	6	—	—	—	—	—	—	—	I 13	—	—	—	—	E 7/A→T	—	Methyl	K-12/G→T	—
22	9	—	—	—	Methyl	—	—	Methyl	—	—	—	—	—	—	—	—	—	—
23	9	—	—	—	Methyl	—	—	—	—	—	—	—	—	—	—	Methyl	—	Methyl
24	4	—	—	—	Methyl	—	—	—	—	—	—	—	—	—	—	—	—	Methyl
25	7	—	—	—	Methyl	—	—	—	—	—	Methyl	—	—	—	—	—	—	—
26	9	—	—	—	Methyl	—	—	Methyl	I 14	—	—	—	—	—	—	Methyl	—	Methyl
27	9	—	—	—	Methyl	—	—	—	I 14	—	—	—	—	—	—	—	—	Methyl
28	7	—	Del	—	Methyl	Del	—	—	—	—	—	Methyl	—	—	—	—	—	—
29	7	—	—	—	Methyl	—	—	—	—	—	—	—	—	—	—	Methyl	—	Methyl
30	9	—	—	—	Methyl	—	—	—	—	—	—	—	—	—	—	—	—	Methyl
31	5	—	—	—	Methyl	—	—	—	—	—	—	—		—	—	—	—	Methyl
32	5	—	—	—	—	—	—	—	—	—	—	—	—	E 4/G→A	—	Methyl	K-12/G→T	Methyl

Prom, promoter; Methyl, methylation; Del, homozygous deletion; Mut, mutation; E, exon; I, intron; K, K-*ras*; H, H-*ras*.

specimens, with 4 of these tumors also exhibiting codon 12 mutations in K-*ras* (Table I and Fig. 3) and 3 cases having detectable *p53* mutations (nos 15, 21, and 32). The *GST-P* promoter was hypermethylated in 23 (72%) cases. Five samples revealed concurrent promoter hypermethylation of both *O6-MGMT* and *GST-P*, but the methylation status of 18 others was independent. Among the 5 cases with methylation of both *O6-MGMT* and *GST-P*, 3 tumors (nos 2, 7, and 32) showed K-*ras* mutations in codon 12; as mentioned above, sample no. 32 interestingly showed mutations in both *p53* (exon 4: G→A) and K-*ras* (codon 12: G→A).

Methylation in the corresponding normal tissues No methylation was detected in the normal tissues except for *p16INK4a* exon 2 (Fig. 4). In *p16INK4a* exon 2, only one case (case 32) was unmethylated, and the remaining 7 cases were methylated. The other genes, including *p14ARF*, *p16INK4a* promoter, *RB1*, *p27Kip1*, *O6-MGMT* and *GST-P*, were not methylated in the corresponding normal tissues.

Correlation between histological grades and gene status With respect to the histological grading of the tumors, we found no significant correlation to gene methylation and/or deletion/mutation among the genes examined. However, tumors with hypermethylated *O6-MGMT* and *GST-P* tend to have higher Gleason scores. Six tumors revealed Gleason scores of 5, 7, 8, 9, 9 and 9, respectively. Promoter hypermethylation of *O6-MGMT* with G-to-A

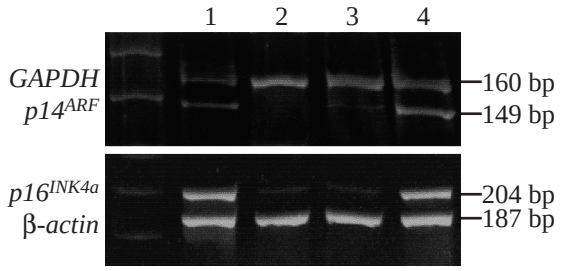

Fig. 1. Differential PCR in the assessment of *p14ARF* and *p16INK4a* homozygous deletions in prostate carcinomas. Cases 1 and 4 have a normal gene status. Cases 2 and 3 show *p14ARF* and *p16INK4a* co-deletions.

transition in K-*ras* gene showed Gleason score 8 or 9 (Table I).

DISCUSSION

Development and progression of prostate carcinoma are thought to be due to the accumulation of both genetic and epigenetic changes.[23] Recent evidence, however, seems to favor epigenetic mechanisms as the main force driving progression of human malignancies. There are many reports concerning prostate cancer progression which imply that unknown epigenetic mechanisms are active.[1]

Genes involved in cell cycle regulation and DNA repair are silenced by hypermethylation of promoter regions, and a number of genes are known to be aberrantly methylated in cancer development. For example, methylation-induced

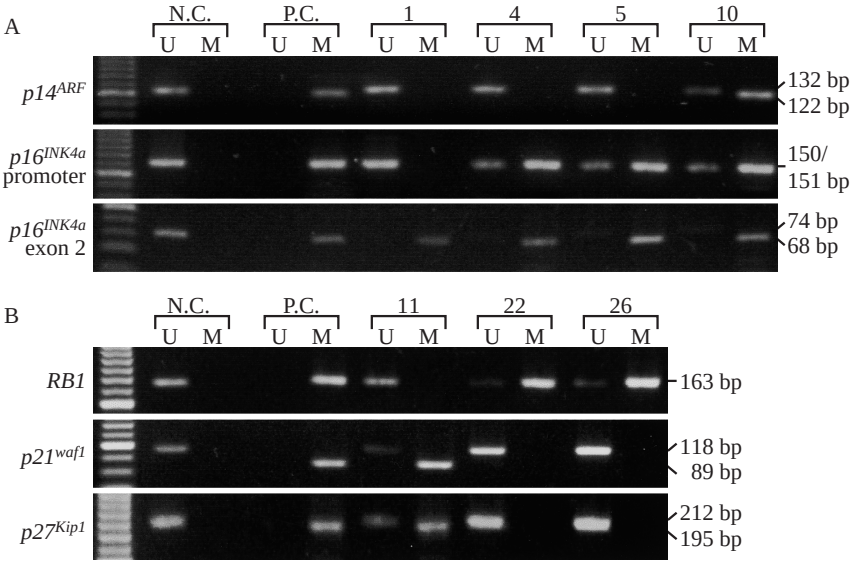

Fig. 2. Methylation-specific PCR of CpG islands in $p14^{ARF}$, $p16^{INK4a}$, *RB1*, $p21^{Waf1}$ and $p27^{Kip}$ genes in prostate carcinomas. A 25-bp DNA ladder (Invitrogen Corp., Carlsbad, CA) is shown on the left as a molecular weight marker. The presence of a visible PCR product in lanes U indicates the unmethylated genes: the presence of product in lanes M indicates the methylated genes. Case numbers are indicated above each gel. (A) In case 10, $p14^{ARF}$ and $p16^{INK4a}$ methylation (M) was detected. In case 1, only unmethylated DNA (U) was apparent, except for exon 2 of the $p16^{INK4a}$ gene. In cases 4 and 5, $p14^{ARF}$ appeared to be unmethylated, whereas $p16^{INK4a}$ DNA was methylated in these tumors. (B) Both $p21^{Waf1}$ and $p27^{Kip}$ showed methylation in case 11, whereas the *RB1* gene is not methylated in this case. In cases 22 and 26, only the *RB1* gene was methylated. NC, normal control DNA from a normal blood; PC, positive control for methylated DNA.

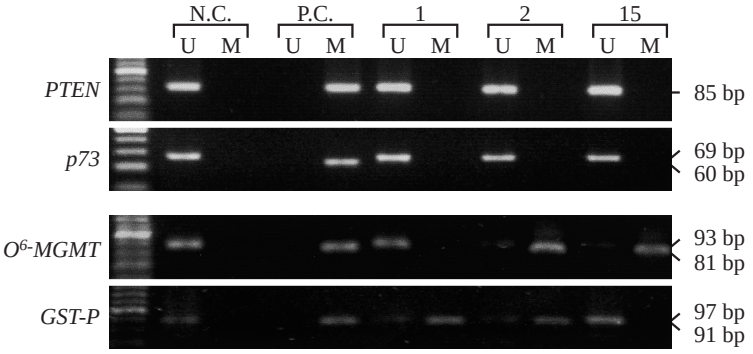

Fig. 3. Methylation-specific PCR of *PTEN*, *p73*, O^6-*MGMT* and *GST-P* promoter regions in prostate carcinomas. Hypermethylation of *PTEN* and *p73* genes was not detected in any case. In sample 1, only the unmethylated base is present in O^6-*MGMT*, while methylated bases are observed for *GST-P*. In sample 2, methylated bases are detected in both O^6-*MGMT* and *GST-P*. In sample 15, methylated bases are detected in O^6-*MGMT*, but not in *GST-P*.

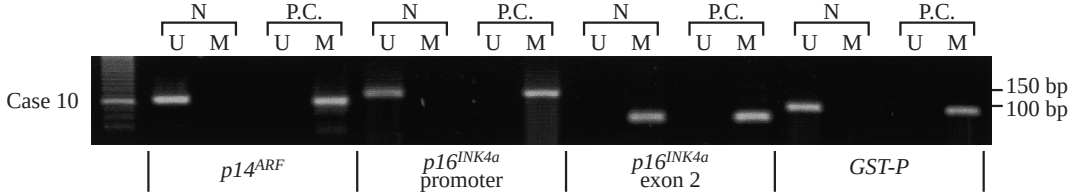

Fig. 4. Methylation-specific PCR of *p14*^{ARF}, *p16*^{INK4a} and *GST-P* genes in the corresponding normal tissues of case 10. Promoter hypermethylation of *p14*^{ARF}, *p16*^{INK4a} and *GST-P* was not detected, but methylated DNA was apparent for exon 2 in the *p16*^{INK4a} gene.

silencing of *p14*^{ARF} and *p16*^{INK4a} has been found frequently in some types of tumors, including gliomas, colon and esophageal carcinomas,[9, 13, 18, 24] while *p14*^{ARF} methylation appears to be rare in lymphomas[24–26] and in pancreatic and hepatic carcinomas.[24, 26] This suggests that silencing by methylation of *p16*^{INK4a}, rather than of *p14*^{ARF}, is likely to be the predominant event in the *INK4a/ARF* (*p14*^{ARF}/*p16*^{INK4a}) locus on chromosome 9p21 in human cancers. In this study, the *p16*^{INK4a} and *p14*^{ARF} genes were co-deleted in 4 cases (~13%) and co-methylated in the promoter regions in only 1 case (3%). The total incidence of deletions and promoter methylation seems to be infrequent; however, these alterations do occur simultaneously in prostate carcinomas, and the combined effects of homozygous deletion and methylation of *p14*^{ARF} and/or *p16*^{INK4a} might function to deregulate both the *RB1* and *p53* pathways.

p14^{ARF} plays a major role in the *p53* pathway by binding specifically to MDM2, resulting in stabilization of both *p53* and *MDM2*.[27] Recently, Esteller *et al.* reported that *p14*^{ARF} silencing by promoter hypermethylation mediates abnormal intracellular localization of MDM2.[24] *p53* mutations may occur more rarely in tumors with inactivation of the *INK4a/ARF* locus than in tumors with the wild-type genes,[28] and reciprocal alterations between *p53* and *p14*^{ARF} have been observed. This pattern may apply to some of the prostate carcinomas in our series. In cases where alterations of *p14*^{ARF} occurred early in the development of the cancer, the tumors may be able to retain wild-type *p53*.

Exon 2 of *p16*^{INK4a} was frequently (21/32 or ~66%) methylated in the prostate carcinomas examined in this study. In addition, we found methylation in exon 2 of the gene in the corresponding normal tissues as well as cancerous tissues. Nguyen *et al.*[29] previously reported a high incidence of *p16*^{INK4a} exon 2 methylation in both normal and cancer areas. Using whole prostate specimens, methylation patterns of *p16*^{INK4a} exon 2 in our previous study were different from those of the other genes examined, in that methylation seemed to be an all-or-nothing event.[30] The underlying mechanisms, however, are not clear because hypermethylation of *p16*^{INK4a} exon 2 was not correlated with p16^{INK4a} expression, as demonstrated by immunohistochemistry.[30] The expression of p16^{INK4a} was occasionally found in a tumor with hypermethylation of *p16*^{INK4a} exon 2, which correlated with upregulated *p16*^{INK4a} transcripts, suggesting complex functions for DNA methyltransferase in prostate tumorigenesis. Transcriptional inactivation may be responsible for this phenomenon.

Recently, *p21*^{Waf1} has been found to be significantly expressed in prostate cancers, but not normal prostate, and the expression of p21^{Waf1} did not correlate with expression of wild-type *p53*.[31] On the other hand, the loss of expression of both p21^{Waf1} and p27^{Kip1} has been associated with metastases in recurrent prostate carcinoma.[32] Although the expression of p21^{Waf1} and p27^{Kip1} was not evaluated in the current study, methylation and loss of function of such genes appears to be insignificant and independent of *p53* status.

With regard to *PTEN* and *p73*, we did not detect any hypermethylation in the prostate carcinomas examined. Several studies have reported frequent *PTEN* mutations in prostate cancers, but there is no direct proof of silencing by methylation since the promoter and enhancer regions of this gene have not yet been defined.[1, 33, 34] Silencing of *p73* in acute lymphatic leukemia and in Burkitt's lymphoma occurs through methylation of the untranslated exon 1 of the gene[10]; in contrast, *p73* methylation was not observed in a survey of tumor cell lines, or in breast, renal and colon cancers.[26]

Hypermethylation of *GST-P* is frequently observed in prostate carcinomas and commonly precedes genome-wide hypermethylation.[35, 36] The loss of GST-P expression through hypermethylation occurs even in prostatic intraepithelial neoplasia, the earliest stage of tumorigenesis.[37] GST-P expression is characteristic of many steroid-dependent neoplasms, such as those of the breast, liver and prostate.[35, 38] It has a wide distribution pattern similar to that of the DNA repair genes *MGMT* and *DAPK*. Concurrent methylation of *GST-P* and *O*^{6}*-MGMT* was detected in 6 of 32 (19%) cases in this study. These 6 tumors showed a higher Gleason score except for one case. Prostate cancer with a Gleason score over 7 tends to have a worse prognosis. Therefore, such genetic conditions may correlate to the tumor prognosis.

It is of particular interest that *O*^{6}*-MGMT* hypermethylation was often associated with mutations in *ras* genes and the few tumors showing both alterations also showed a

higher Gleason score. A strong association was found between *O⁶-MGMT* hypermethylation and the presence of G→A mutations in the K-*ras* gene in colon carcinomas.[14] We also found that *O⁶-MGMT* methylation was not infrequently associated with a mutation in *p53* (3/8 samples). The previous finding that spontaneous G:C→A:T transitions are detected more frequently in the adenine phosphoribosyl transferase (*aprt*) gene of Chinese hamster ovary cells lacking *O⁶-MGMT* activity (28%) than in those expressing *O⁶-MGMT* (5%)[39] suggests that the epigenetic alteration of *O⁶-MGMT* by promoter methylation may preferentially lead to G:C→A:T transitions in transformation-associated genes in human neoplasms. However, in *p53* mutation, only one tumor had a G-to-A transition in these 3 tumors. Because of the relatively small sample size, this is inconclusive. This might be due to less *p53* mutation in prostate carcinogenesis.[20, 23] It is clear from the present data that there is heterogeneity in the methylation profiles of prostate carcinomas. The fact that different combinations within a subset of multiple genes can be methylated in one cell type suggests that methylation is stochastic and provides the cell with a growth advantage. Our results also indicate that alterations in $p14^{ARF}$ and/or $p16^{INK4a}$ can operate independently of *RB1* or *p53* pathways, and that carcinomas with *O⁶-MGMT* hypermethylation may be correlated to G:C→A:T transitions in the *ras* gene and high Gleason scores.

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