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\(^{\text{INK4a}}\), O\(^{\text{6}}\)-MGMT, and GST-P were found to be the most frequently methylated (66%, 25% and 75% of cases, respectively), while methylations of p14\(^{\text{ARF}}\), RB1, p21\(^{\text{Waf1}}\), and p27\(^{\text{Kip1}}\) were far less common (3%, 6%, 6% and 6% of cases, respectively). Methylation of O\(^{\text{6}}\)-MGMT and GST-P genes was defective in about 19% of the cases and there were occasional simultaneous deletions and methylations of p14\(^{\text{ARF}}\) and p16\(^{\text{INK4a}}\) genes (13% and 3% of cases, respectively). In p16\(^{\text{INK4a}}\), methylation occurred in the promoter region in 9% of samples and in exon 2 in 66% of tumors. Hypermethylation of O\(^{\text{6}}\)-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\(^{\text{6}}\)-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. 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\(^{\text{ARF}}\), p15\(^{\text{INK4b}}\), p16\(^{\text{INK4a}}\), GST-P, E-cadherin and VHL genes have been well described. 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). Differential PCR and PCR-SSCP analyses to look for hypermethylation and mutations in multiple genes implicated in prostate cancers, such as p14\(^{\text{ARF}}\), p16\(^{\text{INK4a}}\), RB1, p21\(^{\text{Waf1}}\), p27\(^{\text{Kip1}}\), PTEN, p73, p53, O\(^{\text{6}}\)-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. The equivalent portions of tissues were frozen at −70°C for later DNA extraction as described previously.

Methylation-specific PCR DNA methylation patterns in the CpG islands of the p14\(^{\text{ARF}}\), p16\(^{\text{INK4a}}\), RB1, p21\(^{\text{Waf1}}\), p27\(^{\text{Kip1}}\), PTEN, p73, O\(^{\text{6}}\)-MGMT and GST-P were determined by MSP. 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
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.\(^8,10\) 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 followed by ethanol precipitation, and was resuspended. Control methylated (Intergen) and unmethylated DNA 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 GCC GCG TTG TCG-3′ (sense) and 5′-GCC GCC CCG AAA ACC CGA ACG-3′ (antisense) for the methylated reaction; 5′-TTG TTT GTT GTG TTC TTG TTT GTT T-3′ (sense) and 5′-ACC ACC ACA CCA AAA ACCCAA 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.\(^11\) Duplex PCR amplification was performed to generate a 149 bp fragment covering exon 1B of the p14\(^{ARF}\) gene and a 204-bp exon fragment of p16\(^{INK4a}\) 1A, 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 unmethylated control 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→A transversion in exon 4 of p53.

**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). Methylation 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\(^{INK4a}\).

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

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**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 intrinsic 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%)
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. 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.

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 p14ARF, p16INK4a, RB1, p21Waf1 and p27Kip1 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, p14ARF and p16INK4a methylation (M) was detected. In case 1, only unmethylated DNA (U) was apparent, except for exon 2 of the p16INK4a gene. In cases 4 and 5, p14ARF appeared to be unmethylated, whereas p16INK4a DNA was methylated in these tumors. (B) Both p21Waf1 and p27Kip1 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, O6-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 O6-MGMT, while methylated bases are observed for GST-P. In sample 2, methylated bases are detected in both O6-MGMT and GST-P. In sample 15, methylated bases are detected in O6-MGMT, but not in GST-P.]

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silencing of p14ARF and p16INK4a has been found frequently in some types of tumors, including gliomas, colon and esophageal carcinomas,\(^1\) while p14ARF methylation appears to be rare in lymphomas,\(^2\) and in pancreatic and hepatic carcinomas.\(^3\) This suggests that silencing by methylation of p16INK4a, rather than by p14ARF, is likely to be the predominant event in the INK4a/ARF (p14ARF/p16INK4a) locus on chromosome 9p21 in human cancers. In this study, the p16INK4a and p14ARF 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 p14ARF and/or p16INK4a might function to deregulate both the RB1 and p53 pathways.

p14ARF plays a major role in the p53 pathway by binding specifically to MDM2, resulting in stabilization of both p53 and MDM2.\(^2\)\(^3\) Recently, Esteller et al. reported that p14ARF silencing by promoter hypermethylation mediates abnormal intracellular localization of MDM2.\(^2\)\(^4\) p53 mutations may occur more rarely in tumors with inactivation of the INK4a/ARF locus than in tumors with the wild-type genes,\(^2\)\(^5\) and reciprocal alterations between p53 and p14ARF have been observed. This pattern may apply to some of the prostate carcinomas in our series. In cases where alterations of p14ARF occurred early in the development of the cancer, the tumors may be able to retain wild-type p53.

Exon 2 of p16INK4a 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 in cancers. Nguyen et al.\(^2\)\(^6\) previously reported a high incidence of p16INK4a exon 2 methylation in both normal and cancer areas. Using whole prostate specimens, methylation patterns of p16INK4a 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.\(^2\)\(^7\) The underlying mechanisms, however, are not clear because hypermethylation of p16INK4a exon 2 was not correlated with p16INK4a expression, as demonstrated by immunohistochemistry.\(^2\)\(^8\) The expression of p16INK4a was occasionally found in a tumor with hypermethylation of p16INK4a exon 2, which correlated with upregulated p16INK4a transcripts, suggesting complex functions for DNA methyltransferase in prostate tumorigenesis. Transcriptional inactivation may be responsible for this phenomenon.

Recently, p21\(^{\text{WAF1}}\) has been found to be significantly expressed in prostate cancers, but not normal prostate, and the expression of p21\(^{\text{WAF1}}\) did not correlate with expression of wild-type p53.\(^2\)\(^9\) On the other hand, the loss of expression of both p21\(^{\text{WAF1}}\) and p27\(^{\text{KIP1}}\) has been associated with metastases in recurrent prostate carcinoma.\(^3\)\(^0\) Although the expression of p21\(^{\text{WAF1}}\) and p27\(^{\text{KIP1}}\) 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.\(^3\)\(^1\)\(^3\)\(^2\) Silencing of p73 in acute lymphatic leukemia and in Burkitt’s lymphoma occurs through methylation of the untranslated exon 1 of the gene;\(^3\)\(^3\) in contrast, p73 methylation was not observed in a survey of tumor cell lines, or in breast, renal and colon cancers.\(^3\)\(^4\)

Hypermethylation of GST-P is frequently observed in prostate carcinomas and commonly precedes genome-wide hypermethylation.\(^3\)\(^5\)\(^3\)\(^6\) The loss of GST-P expression through hypermethylation occurs even in prostatic intraepithelial neoplasia, the earliest stage of tumorigenesis.\(^3\)\(^7\) GST-P expression is characteristic of many steroid-dependent neoplasms, such as those of the breast, liver and prostate.\(^3\)\(^8\)\(^3\)\(^9\) 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 5\textsuperscript{\textprime}O\textprime-MGMT hypermethylation and the presence of G\textrightarrow{}A mutations in the K-ras gene in colon carcinomas.\textsuperscript{10} We also found that 5\textsuperscript{\textprime}O\textprime-MGMT methylation was not infrequently associated with a mutation in p53 (3/8 samples). The previous finding that spontaneous G:C\textrightarrow{}A:T transitions are detected more frequently in the adenine phosphoribosyl transferase (aprt) gene of Chinese hamster ovary cells lacking 5\textsuperscript{\textprime}O\textprime-MGMT activity (28\%) than in those expressing 5\textsuperscript{\textprime}O\textprime-MGMT (5\%)\textsuperscript{9} suggests that the epigenetic alteration of 5\textsuperscript{\textprime}O\textprime-MGMT by promoter methylation may preferentially lead to G:C\textrightarrow{}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.\textsuperscript{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\textsuperscript{ARF} and/or p16\textsuperscript{INK4a} can operate independently of RBl or p53 pathways, and that carcinomas with 5\textsuperscript{\textprime}O\textprime-MGMT hypermethylation may be correlated to G:C\textrightarrow{}A:T transitions in the ras gene and high Gleason scores.

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