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Alterations in Promoter Methylation Status of Tumor Suppressor *HIC1*, *SFRP2*, and *DAPK1* Genes in Prostate Carcinomas

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Hypermethylated genomic DNA is a common feature in tumoral tissues, although the prevalence of this modification remains poorly understood. We aimed to determine the frequency of five tumor suppressor (TS) genes in prostate cancer and the correlation between promoter hypermethylation of these genes and low and high grade of prostate carcinomas. A total of 30 prostate tumor specimens were investigated for promoter methylation status of TS hypermethylated in cancer 1 (*HIC1*), death-associated protein kinase 1 (*DAPK1*), secreted frizzled-related protein 2 (*SFRP2*), cyclin-dependent kinase inhibitor 2A (p16), and O-6-methylguanine-DNA methyltransferase (*MGMT*) genes by using bisulfite modifying method. A high frequency of promoter hypermethylation was found in *HIC1* (70.9%), *SFRP2* (58.3%), and *DAPK1* (33.3%) genes in tumor samples that were examined. The current data show high frequency of hypermethylation changes in *HIC1*, *SFRP2*, and *DAPK1* genes in prostate carcinomas of high Gleason Score (GS).

Introduction

Prostate cancer is a common malignancy and a leading cause of death among men in Western countries (Schulz and Hofmann, 2009). Human cancers including prostate cancer develop by interplay of structural mutation of specific genes and epigenetic alterations of tumor suppressor (TS) genes. Although the molecular mechanisms underlying disease development are not established, epigenetic mechanisms, including DNA methylation, are important ethiological parameters in the malignant transformation and progression of prostate cancer. Aberrant methylation in cytosine-phospho-guanine (CpG) islands of genes involved in cell cycle regulation, DNA repair, differentiation, apoptosis, angiogenesis and metastasis may contributes directly to malignancy in different human cancers (Costello and Plass, 2001). In the last decade, aberrations in DNA methylation patterns have been accepted to be a common feature of human cancer (Paz *et al.*, 2003). Histologically identical tumors may arise through different molecular mechanisms such as instable genome, tumor suppressor gene inactivation, proto-oncogene reactivation, chromosomal rearrangements, instability of the microsatellite DNA resulting from deficient DNA repair, and epigenetic alterations in promoter region of distinct TS genes. Promoter hypermethylation is an important pathway for the repression of gene transcription in tumorigenesis.

In the current study promoter hypermethylation of 5 TS genes; *p16INK4a*, hypermethylated in cancer 1 (*HIC1*), death-associated protein kinase 1 (*DAPK1*), secreted frizzled-related protein 2 (*SFRP2*), and O-6-methylguanine-DNA methyltransferase (*MGMT*) were investigated in prostate cancer patients with different Gleason Score (GS). The *p16INK4a* gene, a cyclin-dependent kinase inhibitor and founding member of *INK4* family, inhibits the association between cyclin-dependent protein kinase 4/6 (*CDK4/6*) and D-type cyclins, and blocks phosphorylation of retinoblastoma 1 (RB) (Chin *et al.*, 1998). *DAPK1* is a multi-domain, calmodulin -regulated serine threonine protein kinase that mediates a range of processes, including apoptosis, autophagy, and tumor invasion (Eldik, 2002; Kuo *et al.*, 2006; Lin *et al.*, 2010). SFRP2 is one of the negative modulators of Wnt-frizzled signal transduction pathway which plays an important role in normal development and oncogenesis (Mii and Taira, 2009). *MGMT* is a DNA repair enzyme which catalyses the transfer of alkylating agents (mainly a methyl group from O^6-methylguanine) in DNA (Zhong *et al.*, 2010).

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TS *HIC1* is an essential gene for mammalian development (Carter *et al.*, 2000) and epigenetically inactivated in some types of human cancer (Wales *et al.*, 1995). Hypermethylation mostly lies between the intron 2 and exon 3 of *HIC1* gene and suppresses age-dependent development of cancer by inactivating p53 (Chen *et al.*, 2005). The gene, located on the distal arm of chromosome 17p13.3, encodes a sequence specific zinc-finger transcriptional factor (Pinte *et al.*, 2004) and has a consensus p53 protein binding site in its promoter (Wales *et al.*, 1995; Britschgi *et al.*, 2006). *HIC1* is transcribed from two promoters and dense methylation of either *HIC1* promoter is associated with complete loss of transcription (Chen *et al.*, 2005; Fleuriel *et al.*, 2009). High density of methylation *HIC1* promoter is associated with aggressiveness of the tumor and poor overall survival (Wales *et al.*, 1995; Nicoll *et al.*, 2001; Hayashi *et al.*, 2001; Rood *et al.*, 2002; Waha *et al.*, 2004).

Structural genetic changes have been widely characterized in prostate carcinoma, while epigenetic changes are poorly reported. We aimed to evaluate the frequency of CpG-island hypermethylation in some cancer-associated TS gene promoters in different grades of prostate tumors which may have potential as a diagnostic and/or prognostic biomarker in the presented results.

Methods

Patient and tumor specimens

This study was approved by the Research Ethics Committees of Faculty of Medicine of Cumhuriyet University. Fresh tumoral tissue samples from 30 patients who underwent transurethral resection and open radical retro pubic prostatectomy in the Department of Urology between January 2006 and December 2010 were used in the current study.

Patient's mean age was 70.23 (51–85) years. Digital rectal examination (DRE) was normal in 12 patients whereas the rest were abnormal. Mean prostate-specific antigen (PSA) value was 71.76 ng/dL. Transurethral resection of prostate was performed in 22 of 30 patients because they had a normal PSA level and normal DRE or they had lower urinary tract symptoms associated with advanced disease or other co-morbidities. Radical retro pubic prostatectomy was performed in 7 of 30 patients. In one patient who underwent radical cystoprostatectomy with muscle invasive bladder carcinoma, pathologic examination revealed prostate cancer. Histopathologic evaluation revealed adenocarcinoma in 29 patients and small cell prostate cancer in 1 patient. Five patients had low grade cancer (GS ≤ 6) and 24 patients had high grade cancer (GS ≥ 7–10). The number of patients who had local disease were 8 and 22 of 30 patients had advanced disease.

Methylation specific-polymerase chain reaction and DNA converting

Sodium bisulfite mediated methylation specific-polymerase chain reaction (MSPCR) technique was used to determine the epigentical alterations in the tumoral tissues. This technique deaminates cytosine base in DNA, while 5-methylcytosine resists this action (Boyd and Zon, 2004; Hayatsu, 2008). MSPCR is a sensitive method that discriminately amplifies and detects a methylated region of interest by using methylation site-specific primers on bisulfite-converted genomic DNA. This deamination leads to the conversion of cytosines into uracil residues, which are recognized as thymines in subsequent PCR amplification, whereas the modified cytosines do not react and are therefore detected as cytosines (Clark *et al.*, 2006). Such primers will only anneal to sequences that are methylated and thus contain 5-methylcytosines that are resistant to conversion by bisulfite. Modified and amplified DNA fragments are detected by reverse hibridization of PCR products with commercially available test strips.

Analysis of methylation status of the promoter region of TS genes

Tumoral samples were histologically graded based on the WHO/ISUP and staged according to the TNM classification and examined for tissue specific epigenetic alterations. All fresh specimens were used for detecting possible epigenetic modifications in TS genes. After total genomic DNA isolation genes were modified by sodium bisulfite modification, methylation patterns were determined and correlated with standard clinico-histopathological parameters. DNA methylation patterns in the promoter CpG islands were determined in tumoral tissue samples by MSPCR following the bisulfite modification technique. Forty-five microliters of isolated DNA were denatured by alkalizer (final volume 0.3 mM) at 55°C for 10 min and modified by sodium bisulfite (5.20–5.69 M, pH 5.0; Viennalab) for 4 h at 55°C in the dark. After incubation, binding buffer was added (300 μL per sample) and lysate was transferred into a receiver tube with spin filter, centrifuged at 13 000 rpm for 30 s. Filtrate was discarded, wash buffer (600 μL per sample) was added into the spin filter and centrifuged at 13 000 rpm for 30 s. A mixture of alkalizer ethanol (1:10) was added into the spin filter, 300 μL per sample, then incubated at room temperature for 30 min. After incubation, spin filters were washed again. Elution buffer (30 μL) was added into spin filters, incubated 3 min at room temperature, centrifuged at 13 000 rpm for 1 min, and filters discarded. The resulting filtrate was keep at −20°C. Aliquots of 5 μL of bisulfite modified DNA were used for MSP reactions. Primers for a methylated and unmethylated promoter of the target TS genes (*SFRP2*, p16, *DAPK1*, *HIC1, and MGMT*) were used and multiplex PCR based amplification procedure (Viennalab) was performed for *in vitro* gene amplification. Multiple PCR products were carried out in a Bioer XP thermal cycler after 15 min at 94°C for Pre-PCR, 45 s at 94°C for denaturation, 45 s at 66°C for annealing, and 45 s at 72°C for polymerization of 45 cycles with final extension for 7 min at 72°C. PCR products that amplified from modified DNA from tumoral tissue (10 μL of each PCR reaction) were compared in reverse hybridization strip assay technique (ViennaLab), (Ozdemir *et al.*, 2010). One signal was evaluated as heterozygous inactive (inactivation of one allele); double signals were homozygous hypermethylated (inactivation of both alleles), and samples without any signals were evaluated as hypomethylated (fully active gene). For statistical evaluation, the SPSS 14.0 for Windows was used. The parameters of the patients are presented as mean ± standard deviation and $p < 0.05$ was considered statistically significant.

Results

By MSPCR technique, we evaluated the methylation status for *HIC1*, *SFRP2*, *DAPK1*, and *MGMT* TS genes in prostate tumors with different GSs. We found that *HIC1* were hypermethylated in 70.1% (17/24) in prostate adenocarcinomas with high GSs.

Clinicopathologic data and follow-up knowledge

A total of 30 male patients with mean age 70.23 with prostate carcinomas were clinically diagnosed and treated. Solid tumoral tissues (1 small cell prostate carcinoma [SCPC] and 29 adenocarcinoma) were examined in the current study. The number of patients investigated according to stages of tumors were; 12 in pT1, 8 in pT2, and 10 in pT4. GS was between 6 and 8 in patients with pT1, 8 in patients with pT2, and 9 or 10 in all patients with pT4 tumors (Table 1).

Increased percentage of promoter hypermethylation in *HIC1, SFRP2,* and *DAPK1* genes and the clinical and histopathological parameters in prostate cancer of 30 samples showed a high sensitivity and specificity. There was no promoter hypermethylation-gene inactivation in TS p16 in all tumor samples examined (100% of tumors were hypomethylated). Tumor specimens showed high frequency of promoter hypermethylation in *HIC1* (70.9%), *SFRP2* (58.3%), and *DAPK1* (33.3%) genes.

The *HIC1* gene was fully inactive (hypermethylated) in one case of SCPC but the rest of studied genes were fully active (100%) in that case (Table 2). In general, various (heterogeneous type) epigenetic alterations were detected for some TS genes in distinct prostatic adenocarcinomas of different GS (Tables 1 and 2). TS *DAPK1* (33.3%) and *MGMT* (8.3%) showed partial and/or full inactivation in early and/or late stage tumors (Table 2), (Fig. 1). High frequency of full promoter hypermethylation (homozygous for both alleles) for *HIC1* (70.9%) and *SFRP2* (58.3%) genes were detected in prostate tumor specimens that were examined. Two groups of tumors with different GS (Group 1, GS ≤ 6 and Group2, GS ≥ 7) were statistically compared. While *HIC1* hypermethylation profiles were significant for both groups, only *SFRP2* gene showed statistical significance for GS ≥ 7 in the current tumoral tissues ($p > 0.016$, Table 2). No difference was found between GS for the rest of the genes that were examined ($p > 0.016$, Table 2).

Discussion

Promoter hypermethylation which repress transcription of the TS genes leading to gene silencing has been extensively studied. Epigenetic alterations can disrupt TS gene function as an alternative to inactivating genetic mutations (Jones and Baylin 2002; Brophy *et al.*, 2003; Zhang *et al.*, 2006; Marsit *et al.*, 2006a; Schulz and Hofmann, 2009). Recent developments in epigenetic methodologies and gene silencing techniques opened a new area for the identification of epigenetic parameters (Marsit *et al.*, 2006b; Nojima *et al.*, 2007; Shih *et al.*, 2007; Hoffmann *et al.*, 2007; Awakura *et al.*, 2008). Although the mechanisms of specific gene hypermethylation in cancer precursor cells are unknown, locus-specific *de novo* hypermethylation may occur in those cells by hyperactivity of DNA methyltransferase enzyme as claimed by Simon and Lange (2008).

In the present study, we determined the hypermethylated profiles for promoter regions of target TS genes *HIC1*, p16, *MTMG*, *DAPK1*, and *SFRP2* in prostate cancers. *SFRP1* is a marker of higher tumor stage, grade, and poorer survival in renal cell carcinoma (Dahl *et al.*, 2007). Marsit *et al.*, (2005) have reported methylation-induced epigenetic alterations in *SFRP* genes associated with the occurrence and/or progression of bladder cancer. In our previous study, full inactivation of *SFRP2* was reported in two cases of fistula associated mucinous type anal adenocarcinomas (Sen *et al.*, 2010). SFRPn proteins are also responsible for the constitutive inactivation of WNT signaling (Wolff *et al.*, 2005). Hypoexpression due to hypermethylated *SFRP2* was reported in 94.2%, 52.4%, 37.5%, and 16.7% of patients with colorectal carcinoma (CRC), adenocarcinomas, hyperplastic polyps, and ulcerative colitis, respectively (Huang *et al.*, 2007). *HIC1* encodes a zinc-finger transcription factor that is essential for mammalian development (Esteller, 2000). HIC1 forms a transcriptional repression complex with NAD-dependent deacetylase sirtuin-1 (*SIRT1*) deacetylase, and this complex directly binds the *SIRT1* promoter and represses its transcription. The inactivated form was reported in a few types of human cancer (Berezovska *et al.*, 2006) and is epigenetically inactivated, but not mutated, in some human cancers (Van Leenders *et al.*, 2007). Inactivation of *HIC1* results in upregulated SIRT1 expression in normal or cancer cells; this deacetylates and inactivates p53, allowing cells to bypass apoptosis and increases cancer risk in mammals.

Table 1. Clinical Characteristics of the Patients with Prostate Carcinomas

Tumor type (*n*/%)		Adenocarcinoma: 29/96.6%
		Small cell prostate cancer: 1/3.33%
The mean age (Min-Max)		70.23 ± 8.43 (51–85)
Operation type (*n*/%)		TUR (P) 22/73%
		Radical prostatectomy 8/27%
Pre-op mean serum PSA values		71.76 ± 23.94 (0.003–1237) ng/dL
Gleason scores (*n*/%)	Low grade (Gleason score: ≤6)	5 (16.7%)
	High grade (Gleason score: ≥7–10)	24 (80%)
	SCPC	1 (3.33%)

PSA, prostate-specific antigen; SCPC, small cell prostate carcinoma.

Table 2. The Percentage, Gleason Scores (grade), Histopathological Evaluation, and Promoter Methyl Status of Target Tumor Suppressor Genes of the Adenocarcinomas Investigated in the Patients with Prostate Cancer

		WHO/ISUP–Tumor type total = 30						
				Adenocarcinoma n = *29*				
Gene	*Epigenetic profile*	*SCPC* n = *1*		*Group 1 GS ≤6* (n = *5*)		*Group 2 GS ≥7–10* (n = *24*)		p
SFRP2 (*n*/%)	Unmethylated active gene	1	100	5	100	9	37.5	0.016
	Partially methylated	-	-	-	-	1	4.2	
	Fully methylated inactive gene	-	-	-	-	14	58.3	
p16 (*n*/%)	Unmethylated active gene	1	100	5	100	24	100	
	Partially methylated	-	-	-	-	-	-	
	Fully methylated inactive gene	-	-	-	-	-	-	
DAPK1 (*n*/%)	Unmethylated active gene	1	100	5	100	15	62.5	$p > 0.05$
	Partially methylated	-	-	-	-	1	4.2	
	Fully methylated inactive gene	-	-	-	-	8	33.3	
HIC1 (*n*/%)	Unmethylated active gene	-	-	1	20	5	20.8	$p > 0.05$
	Partially methylated	-	-	1	20	2	8.3	
	Fully methylated inactive gene	1	100	3	60	17	70.9	
MGMT(*n*/%)	Unmethylated active gene	1	100	5	100	22	91.7	$p > 0.05$
	Partially methylated	-	100	-	-	-	-	
	Fully methylated inactive gene	-	100	-	-	2	8.3	

GS, Gleason score.

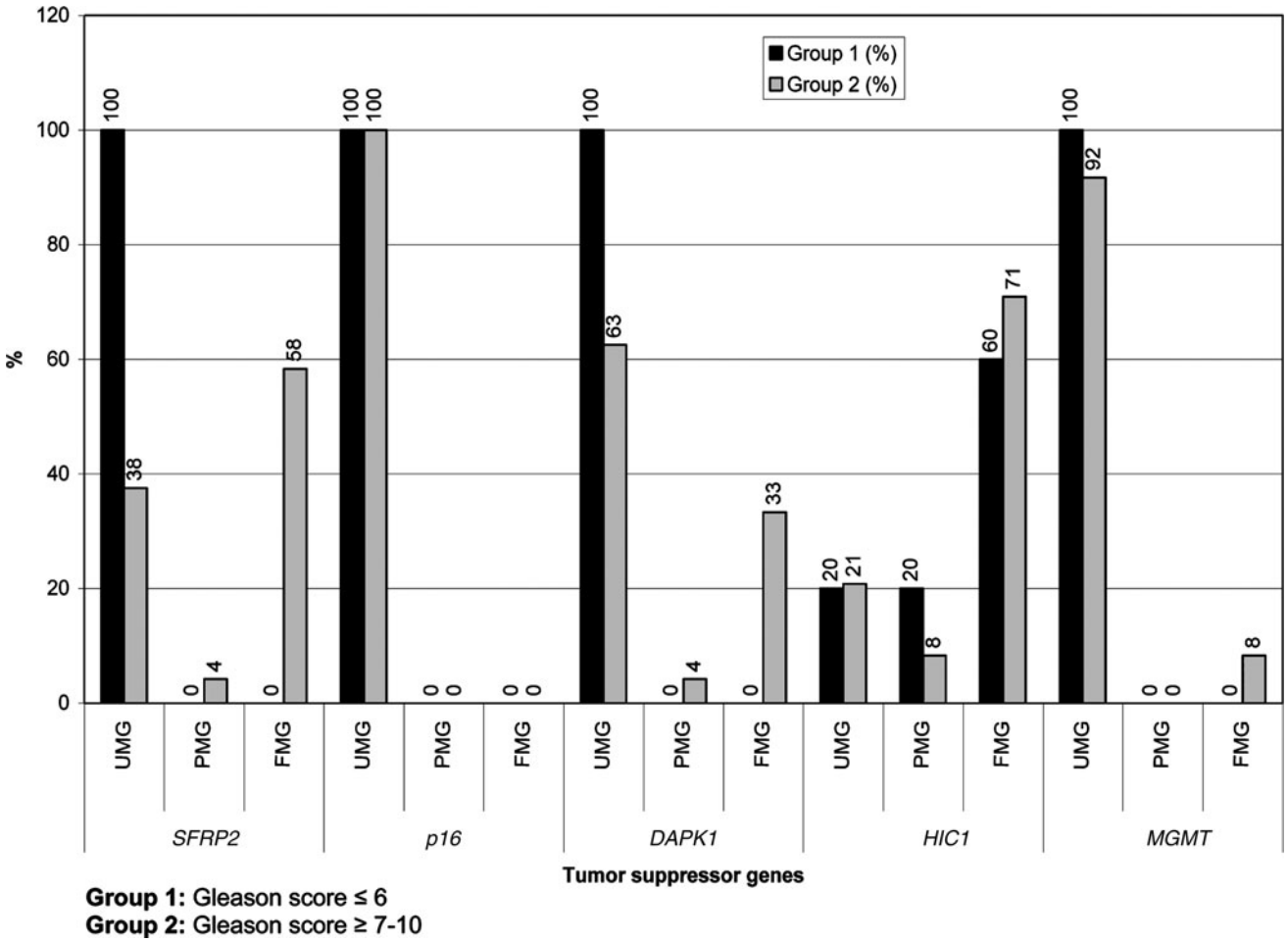

FIG. 1. Bar graphs show the methylation ratios of target tumor suppressors studied in the current patients. UMG, unmethylated gene; PMG, partially methylated gene; FMG, fully methylated gene.

In the current study we aimed to find hypermethylation frequency of the 5 target TS genes and to correlate this data with clinical findings. Results show high frequency of promoter CpG methylation of various TS genes (*HIC1*, *SFRP2*, and *DAPK1*) in prostate tumors and their association with high Gleason grade. These data might suggest a role for *HIC1* in prostate carcinogenesis and indicate that *HIC1* promoter methylation might be a diagnostic and prognostic biomarker in prostate cancer. In the low GS group (GS ≤ 6; $n = 5$) only *HIC1* was hypermethylated ($n = 1$, 20% partially methylated and $n = 3$, 60% fully methylated). In the high GS group (GS ≥ 7; no = 24) the *HIC1* gene was also hypermethylated ($n = 2$, 8.3% partially and $n = 17$, 70.9 fully hypermethylated). Morton *et al.* (1996) have showed that the hypermethylation of chromosome 17p locus D17S5 is a tissue-specific event in prostate DNA, and they hypothesized that methylation of this and/or related loci may play a role in the extreme predilection of this gland to neoplastic growth. Only the *SFRP2* gene showed statistically significant relationship with GS ≥ 7 tumors ($p > 0.016$, Table 2). Different frequency of hypermethylation profiles were detected for the other TS genes in both groups but the results were not statistically significant (Table 2). Results confirmed the association between gene hypermethylation status and advance tumoral differentiations. The current results were also supported by other findings (Catto *et al.*, 2005; Kim and Kim, 2009). They found that promoter hypermethylation was present in 86% of transitional cell carcinoma and also occurred more frequently and more extensively in urinary tract tumors (94%) than in bladder tumors (Catto *et al.*, 2009). No correlation was found between p16, *MGMT* gene inactivation and tumor grade, stage, recurrence, progression and/or invasion. Tumoral specimens showed fully methylated profiles for *HIC1* (70.9%), *SFRP2* (58.3%), and a partially hypermethylated profile for the *DAPK1* (33.3%) and *MGMT* (8.3%) genes in different ratios according to the tumor grade (Table 2, Fig. 1). The current data also shows a high frequency of hypermethylation changes in the *HIC1* and *SFRP2* TS genes. *DAPK1* gene, a positive regulator for apoptosis, showed promoter hypermethylation frequency at 33.3% for the high GS group in the prostate cancer samples.

HIC1 is a TS gene which is epigenetically inactivated in many human cancers and has a central role in the DNA damage response through the establishment of several complex regulatory loops involving p53, *SIRT1* and *E2F1* (Britschgi *et al.*, 2006; Tseng *et al.*, 2009). As shown by Naqvi *et al.*, (2010) and Van Rechem *et al.*, (2010) the p53-binding sequence lies in a region of the *SIRT1* (SIRTUIN1) promoter that also binds the transcriptional repressor *HIC1* tumor suppressor. Mohammad *et al.*, (2011) have shown that the homozygous deletion and/or inactivation of *HIC1* in mice results in major developmental defects and embryonic lethality. The same results were also reported by Dehennaut and Leprince, (2009). They showed that all copies of *HIC1* are completely unmethylated and ubiquitously expressed in normal mammalian tissues (Dehennaut and Leprince, 2009). Fleuriel *et al.*, (2009) proposed that epigenetical inactivation of *HIC1* might "addict" cancer cells to altered survival and signaling pathways during the early stages of tumorigenesis by cooperating within a complex of *HIC1*-p53-*SIRT1* regulatory loop. A *HIC1*-*SIRT1*-p53 circular loop in which hypermethylation of *HIC1* represses the transcription of *SIRT1* that deacetylates and inactivates p53 thus leading to *HIC1* inactivation has been identified in cell and animal models. Hypermethylated *HIC1* is an epigenetically regulated transcriptional repressor that functionally cooperates with p53 to suppress age-dependent development of cancer in mice (Chen *et al.*, 2005). Tseng *et al.*(2009) proposed that deregulation of the *HIC1*-*SIRT1*-p53 loop in lung cancer patients is linked to poor prognosis. Yegnasubramanian *et al.*, (2004) claimed that the aberrant DNA hypermethylation patterns may be the earliest somatic genome changes in prostate cancer for glutathione S-transferase pi (*GSTP1*), adenomatous polyposis coli (*APC*), *RASSF1*a, *PTGS2*, and multi-drug resistance gene 1 (*MDR1*) genes. The CpG islands for *EDNRB*, *ESR1*, *CDKN*2a, and *hMLH1* genes exhibit low to moderate rates of hypermethylation and the CpG islands for *DAPK1*, *TIMP3*, *MGMT*, *CDKN*2b, p14/ARF, and *CDH1* genes are unmethylated for prostate carcinomas (Yegnasubramanian *et al.*, 2004). Our results showed moderate type hypermethylation for *DAPK1* (33.3%) and hypomethlated profile for *MGMT* gene (8.3%) as reported by Yegnasubramanian *et al.*, (2004).

In conclusion, we have investigated promoter hypermethylation status of six genes, p16, *SFRP2*, *HIC1*, *DAPK1*, and *MGMT* in 30 prostate carcinoma patients. Our results confirm the importance of methylation in the molecular pathogenesis of prostate adenocarcinomas, with the majority of tumors having a high rate of CpG islands methylated in *HIC1*, *SFRP2*, and *DAPK1* gene promoters. Thus, tumoral tissue specific identification needs not only pathological diagnosis, but also oncogenetic and epigenetic analyses, for the correct diagnosis and case specific therapy in prostate carcinoma.

Disclosure Statement

There is no conflict on the current results.

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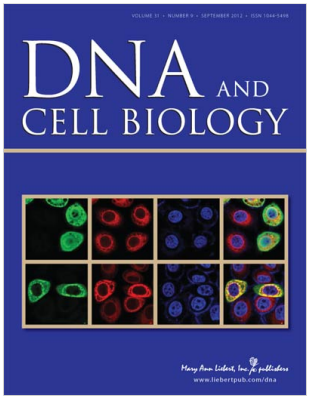

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