Disease Markers 28 (2010) 361–368
DOI 10.3233/DMA-2010-0716
IOS Press

Frequency of *P16INK4a* and *P14ARF* genes methylation and its impact on bladder cancer cases in north Indian population

Seyed Ali Hosseini[a,b,*], Ranbir Chander Sobti[a,*], Kianoosh Malekzadeh[a], Shrawan Kumar Singh[c] and Kusum Joshi[d]

[a]*Department of Biotechnology, Panjab University, Chandigarh, India*
[b]*Department of Medical Genetics, National Institute of Genetic Engineering and Biotechnology, Tehran, Iran*
[c]*Department of Urology, Post Graduate of Medical Education and Research, Chandigarh, India*
[d]*Department of Histopathology, Post Graduate of Medical Education and Research, Chandigarh, India*

Abstract. *Introduction*: Amongst the genitourinary cancers, carcinoma of the urinary bladder is one of the leading causes of death in India. Hypermethylation of the CpG islands of gene promoter is one of the earliest and most frequent epigenetic alterations leading to cancer as well as in its development. Several studies have suggested that tumour suppressor genes play a key role in the development of cancer. Methylation in the *CDKN2A* has been associated with various malignant diseases, but information with respect to urinary bladder cancer is lacking in north Indian population.
Materials and methods: We analyzed the methylation of *P16INK4a* and *P14ARF* in 80 tissues and matched blood samples of patients suffering from bladder cancer and 80 blood samples of cancer-free individuals by MS-PCR.
Results: In tissue and matched blood samples of bladder cancer patients, the incidence of *P14ARF* hypermethylation significantly increased (OR = 0.31, 95%CI = 0.12–0.8, $P = 0.01$) and (OR = 0.0, 95%CI = 0.0–0.62, $P = 0.006$) respectively with an increase in age. Clinicopathological analysis revealed that *P14ARF* hypermethylation in tissue and blood samples was significantly associated with invasive stage ($\geqslant$ T2) (OR = 0.21, 95%CI = 0.08–0.51, $P = 0.0002$) and (OR = 0.09, 95%CI = 0.03–0.37, $P = 0.00001$) respectively. Muscle invasive tumour stage ($\geqslant$T2) showed significant association with increased risk of *P16INK4*α promoter hypermethylation in tissue and blood samples of patients (OR = 0.38, 95%CI = 0.17–0.82, $P = 0.01$) and (OR = 0.13, 95%CI = 0.05–0.36, $P = 0.00005$) respectively.
Conclusion: These results suggest that the CpG island hypermethylation status of the defined panel of genes may be a useful biomarker in patients suffering from bladder cancer.

Keywords: Bladder cancer, methylation, tumour suppressor gene, *P14ARF*, *P16INK4a*

1. Introduction

Bladder cancer (BC) represents 2% of all human malignancies. It is estimated that more than 68,810 new patients were to be diagnosed with BC in the United States in 2008 and 14,100 patients are expected to die of this disease [1]. In India carcinoma of the urinary bladder is one of the leading causes of death, amongst the genitourinary cancers [2]. Several chemical and environmental exposures have been linked to the development of bladder cancer. However, in most cases, it has been difficult to establish a causality relation due to the long period between exposure and development of clinical sings [3]. Cigarette smoking is the most important risk factor for bladder cancer and is thought to be responsible for 1 to 2 of every three newly diagnosed cases of bladder cancer [4].

*Corresponding authors: Seyed Ali Hosseini and Ranbir Chander Sobti, Department of Biotechnology, Panjab University, Chandigarh, 160014, India. Tel.: +91 172 2541409; E-mail: dr.alihosseini@yahoo.com.

ISSN 0278-0240/10/$27.50

About 95% of bladder neoplasms are transitional cell carcinomas. The remainder are squamous tumours, adenocarcinomas, and other subtypes. At the time of diagnosis, $>$ 60% of the transitional carcinomas are papillary noninvasive (Ta), 10–20% show invasion limited to the lamina propria (T1), and 20% present muscle or deeper infiltration (T2-T4) [5]. Bladder cancer is the result of monoclonal genetic changes, and the multiple synchronous or metachronous tumours are derived from micrometastatic foci that have migrated from the original site rather than from a polyclonal mutation [6–8].

Cancer is a polygenetic and polyepigenetic disease [9]. Hypermethylation of the CpG islands of gene promoter is one of the earliest and most frequent epigenetic alterations found in cancer [10,11] as well as its development [12]. It is an important epigenetic mechanism for gene silencing, which may confer tumour cells on growth advantage [10,13]. Many cellular pathways are inactivated by this epigenetic event, including DNA repair (*BRCA1, MGMT*), cell cycle regulation (*p16INK4a, p15INK4a, Rb, p14ARF*), apoptosis (*DAPK, TMS1*), cell adherence, drug resistance, detoxification, differentiation, angiogenesis and metastasis [12,14].

The specific patterns of CpG island hypermethylation between tumour types may provide a useful signature for tumour diagnosis and prognosis [15,16]. Moreover, genes that are frequently methylated in specific tumours have been used as molecular targets for the detection of neoplastic cells in body fluids such as urine and plasma. These genes provide additional targets for noninvasive early diagnosis and monitoring of cancers [17,18].

Tumour suppressor genes prevent cells from malignant transformation. Tumour suppressor genes function by one of the following mechanisms: protect the genome from mutagenic events, impede dysregulated progression through the cell cycle, induce apoptosis in cells that escape normal cell cycle controls, and inhibit cellular migration and metastasis. Classically, tumour suppressor genes have been described to acquire loss of function mutations or deletions leading to their inability to delay malignant transformation. Alternatively, epigenetic events, such as methylation, represent a distinct mechanism of tumour suppressor gene inactivation. Aberrant gene promoter methylation of these genes has been known to be associated with gene silencing and is functionally equivalent to a deleted gene [19].

Application of novel molecular biology techniques has increased our appreciation of the widespread changes in methylation patterns that occur during urinary bladder carcinogenesis. Hypermethylation occur throughout all stages of tumourigenesis, including the early phases, and is increasingly recognized as a major mechanism involved in tumour suppressor genes silencing or inactivation [20].

A large number of genes have been reported to be hypermethylated in bladder cancer. Some examples include the *p16INK4a* (at *CDKN2A*$^{\mathrm{INK4a}}$ locus on chromosome 9p21), E-cadherin (*CDH1*, encoding a transmembrane glycoprotein that modulates calcium-dependent intercellular adhesion), and *RASSF1A* (ras association domain family gene 1, isoform A) genes [21–23]. Inactivation of the *CDKN2A* genetic locus, due to mutations, homozygous deletion and promoter methylation, have also been reported at varying frequencies [24–26]. The well characterized *CDKN2A* locus at 9p21 encodes two unrelated cell cycle inhibitors, *p16INK4a* (referred to as p16 throughout) and *p14ARF* (referred to as ARF throughout) from a partially shared genomic sequence, which function upstream of *Rb* and $p53$, respectively [27]. *P14ARF* and *P16INK4a* are indicated as candidates for hypermethylation-associated inactivation, because they contain documented CpG islands that can be silenced by this epigenetic alteration in several kinds of tumours [24].

In recent studies,*P14ARF* and *P16INK4a* promoter hypermethylation has been reported in many tumours, including gastric [25], breast [26], multiple myeloma [27], Leukemia [28], melanoma [29], and prostate cancer [30].

To determine whether promoter methylation has a useful role in managing urothelial cancer, the present study aimed to detect *P14ARF* and *P16INK4a* promoter methylation by methylation-specific polymerase chain reaction (MS-PCR) from tumour and blood DNA of bladder cancer patients; and analyze the distribution of various clinicopathological parameters and some risk factors. To the best our knowledge, this is the first analysis of *P14ARF* and *P16INK4a* promoter methylation status in bladder cancer patients in Indian population.

2. Materials and methods

2.1. Study subjects

80 histologically confirmed incident bladder cancer speciments and matched blood samples were recruited from Department of Urology, Post Graduate Institute

Table 1
Primer sequences for *P16INK4a* and *P14ARF* methylated and un-methylated

Gene	Primer	Primer sequences	Reference
P14ARF	M-Forward	5‘-GTCGAGTTCGGTTTTGGAGG-3‘	E.P. Xing et al. (1999)
	M-Reverse	5‘-AAAACCACAACGACGAACG-3‘	
	UM-Forward	5‘-TGAGTTTGGTTTTGGAGGTGG-3‘	
	UM-Reverse	5‘-AACCACAACAACAAACACCCCT-3‘	
P16INK4a	M-Forward	5‘-TTATTAGAGGGTGGGGCGGATCGC-3‘	J. G. Herman et al. (1996)
	M-Reverse	5‘-GACCCCGAACCGACCGTAA-3‘	
	UM-Forward	5‘-TTATTAGAGGGTGGGGTGGATTGT-3‘	
	UM-Reverse	5‘-CAACCCCAAACCACAACCATAA-3‘	

of Medical Education and Research, Chandigarh, India. None of the patients had received chemotherapy or radiation before inclusion.

The criteria for the selection of patient were based on clinical proforma, pathological, and histo-pathological records. This study was approved by the Ethical Committee of the Institute. Bladder tumour samples were reviewed by the study pathologist and classified according to the 1973 and 2004 WHO guidelines for bladder tumours (www.uroweb.org). Patients data were collected through interview where demographic features, clinical details, and environmental exposure were recorded using a standard clinical performa. For control group of patients who were admitted in hospital without any prior history of cancer, pre-cancerous lesions or acute inflammatory disease were selected. Cases and controls were matched by age, sex, and socio-economical status. Most of the subjects had completed their primary education.

A signed consent form was obtained from each of the patients. The amount of exposure to dangerous materials and chemicals in work place such as aromatic amines, polycyclic aromatic hydrocarbons and diesel were regarded as occupational exposure. Subjects who were exposed to such material over 5 years were classified as the high risk cases. Smoking habit was also studied among the subjects. Subjects who had smoked at least 100 cigarettes or chewed tobacco 100 times or more during their lifetimes were defined as smokers.

3. Methods

Tissues and matched blood samples from each case and blood samples were obtained from controls. DNA was extracted by a standard phenol/chloroform method. DNA samples were stored at -20°C until analysis.

4. Bisulphite treatment of DNA

P16INK4a and *P14ARF* promoter methylation was analyzed in tumour and matched mononuclear blood cells. DNA methylation patterns in the CpG island of these genes were determined by methylation-specific PCR. Briefly, 1 μg of DNA from tumour and blood cells was denatured by NaOH, and subsequently treated with hydroquinone and sodium bisulfite at 55°C for 16 h. Modified DNA samples were purified using the Wizard DNA purification following the Manufacturer instructions (Promega, USA) and eluted in 50 μl of double distilled water. NaOH was added to complete the modification and followed by ethanol precipitation.

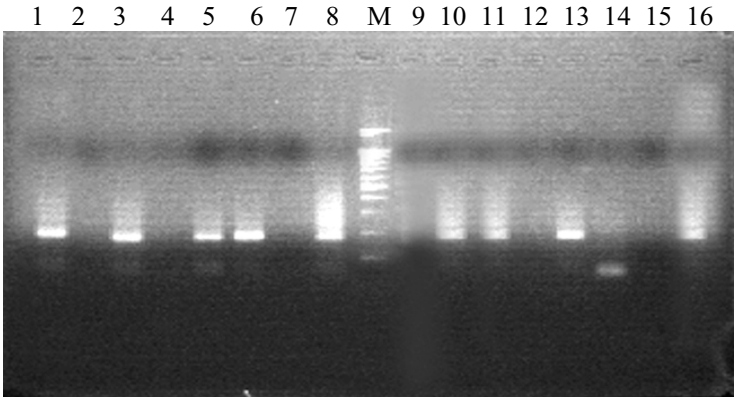
5. Methylation specific PCR (MS-PCR)

Resuspended DNA was used in a PCR reaction. Primer sequences and PCR conditions for *P16INK4a* and *P14ARF* have been reported elsewhere [31,32], Table 1.

The reaction was carried out in a final volume of 50 μL containing 50 ng of bisulphite treated DNA. 1.5 mmol/L of $MgCl_2$, 0.2 mmol/L of each dNTP (Fermentas, USA), 200 μM of each of the primers and 2.5 unit of Taq polymerase (Fermentas, USA). DNA was amplified during 30 cycles with an initial denaturation of 5 minutes at 95°C and a final extension of 5 minutes at 72°C. The cycling program consisted of 30 sec denaturation at 95°C, 30 sec annealing at 61 and 65°C (*P14ARF* and *P16INK4*α) respectively, and 30 sec extension at 72°C. PCR products were electrophoresed on a 2% agarose gel. *P14ARF* methylated and unmethylated were recognized by 160 and 165 bp respectively, while for *P16INK4a* were identified by 150 bp for methylated and 151 bp for unmethylated (Figs 1, 2). In order to avoid misinterpretation of methylated and unmethylated product, reaction mixture for methylated and un-methylated primers were prepared separately.

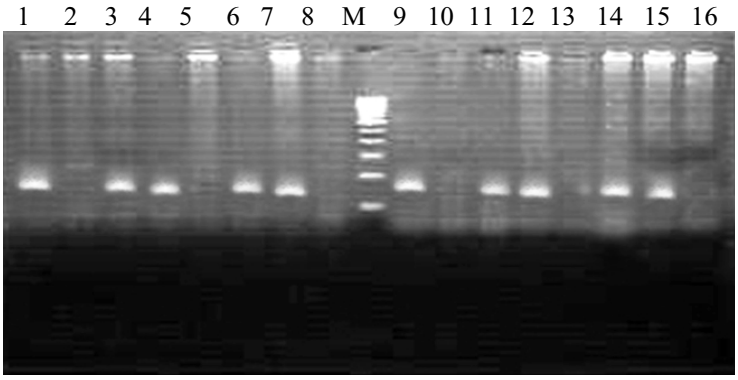
6. Statistical analysis

Statistical analysis was carried out with SPSS 11.5 and EPI info 3.2 software programs. ORs were adjusted



Lane M	:	100-bp marker
Lane1	:	Positive control
Lane2	:	Negative control
Lane3,5,7,9,11,13,15	:	Methylated (160 bp)
Lane4,6,8,10,12,14,16	:	Unmethylated (165 bp)

Fig. 1. Methylation specific PCR for *P14ARF* of tissue samples.



Lane M	:	100-bp marker
Lane1	:	Positive control
Lane2	:	Negative control
Lane3,5,7,9,11,13,15	:	Methylated (150 bp)
Lane4,6,8,10,12,14,16	:	Unmethylated (151 bp)

Fig. 2. Methylation specific PCR for *P16INK4a* of tissue samples.

for confounding factors, such as age, smoking, and socio-economical status. Multivariant analysis, odds ratios, and 95% confidence interval (CI) were used to describe the strength of association.

7. Results

Demographic characteristics of cases and controls are summarized in Table 2. The hypermethylation of *P14ARF* and *P16INK4*α genes were studies carried in 80 patients (69 (86.25%) males, and 11 (13.75%) females) with mean age of 57.42 years. The control group consisted of 71 (88.75%) males and 9 (11.25%) healthy females with mean age of 56.35 years. 8.75% of patients and 32.50% of controls were current smokers and 36.25% of cases and 21.25% of controls had high risk jobs.

No hypermethylation of *P14ARF* and *P16INK4*α were detected in peripheral blood sample of 80 cancer-free individuals.

P14ARF methylation was found in 18 out of 80

Table 2
Demographic characteristic of cases with bladder cancer and healthy controls

Variable	Cases	Controls
Age (year)		
Mean(± SD)	57.42 (± 12.59)	56.35 (± 10.13)
Median range	25–86	30–85
Sex		
–male	69 (86.25%)	71(88.75%)
–female	11 (13.75%)	9 (11.25%)
Smoking		
–current smokers	31(38.75%)	26(32.50%)
–non smokers	49(61.25%)	54(67.50%)
Job		
–high risks	29(36.25%)	17(21.25%)
–low risks	51(63.75%)	63(78.75%)
Alcohol		
–drinkers	35 (43.75%)	29 (36.25%)
–non drinkers	45 (56.25%)	51 (63.75%)
Stage		
$\leqslant$ T1 (superficial)	63(78.75%)	
$\geqslant$ T2 (invasive)	17(21.25%)	

(22.5%) tissue samples of bladder cancer patients. Among 18 cases with *P14ARF* methylation in tumour tissues, MS-PCR was able to detect the same change in the matching blood samples of 8 (44.44%) cases. For the other 62 cases with no detected *P14ARF* methylation in tumour tissue, no signal was obtained on matching blood samples by using MS-PCR.

In tissue and matched blood samples of bladder cancer patients, the incidence of *P14ARF* hypermethylation significantly increased (OR = 0.31, 95%CI = 0.12–0.8, $P = 0.01$) and (OR = 0.0, 95%CI = 0.0–0.62, $P = 0.006$) respectively with an increase in age.

Clinicopathological analysis revealed that *P14ARF* hypermethylation in tissue and blood samples was significantly associated with invasive stage ($\geqslant$ T2) (OR = 0.21, 95%CI = 0.08–0.51, $P = 0.0002$) and (OR = 0.09, 95%CI = 0.03–0.37, $P = 0.00001$) respectively (Table 3).

Of the bladder tumour samples amplified, 32 (40%) presented *P16INK4*α promoter hypermethylation. Among 32 cases with *P16INK4*α methylation in tumour tissues, MS-PCR was able to detect the same change in the matching blood samples of 12(37.5%) cases. For the other 48 cases with no detected *P16INK4*α methylation in tumour tissue, no signal was obtained on matching blood samples by using MS-PCR. Moreover, 41(51.25%) tumour samples presented one of the *P16INK4*α or *P14ARF* hypermethylation.

Table 4 shows the hypermethylation distribution of *P16INK4*α gene along with various clinico-pathological parameters including age, smoking and stage of carcinoma in blood samples the patients. Muscle invasive tumour stage ($\geqslant$ T2) showed significant association with *P16INK4*α promoter hypermethylation in tissue and blood samples of the patients (OR = 0.38, 95%CI = 0.17–0.82, $P = 0.01$) and (OR = 0.13, 95%CI = 0.05–0.36, $P = 0.00005$) respectively.

No association was found between *P14ARF and P16INK4*α methylation with tobacco, high risk jobs and alcohol consuming in bladder cancer patients.

8. Discussion

There is growing evidence that DNA methylation of CDKN2A (INK4a/ARF) is an important mechanism of gene inactivation in cancer and its potential as a molecular marker has been regarded. This study has used methylation – specific PCR for the amplification of P14ARF and P16INK4a promoter regions in tumour tissue as well as matched blood DNA of cancer patients and healthy controls. We found promoter methylation in 47.1% of P14ARF and 58.8% of P16INK4a in the tissue samples of bladder cancer cases with invasive stage. These genes are involved in cell cycle regulation mechanisms [33]. Our finding have shown that methylation of these genes might be related with advanced stage and tumour metastasis. On the other hand, we found that hypermethylation at these genes loci detected in blood samples of patients are related to advanced disease stages. No promoter methylation was found in the blood DNA of control subjects. Detecting the same alteration in blood and tumour DNA indicated that the methylated DNA released into circulation may have derived from the primary tumour. This is confirmed other literature review which evident that methylation can occur in a tissue-specific or cancer-specific manner. Cancer-specific methylation is usually detected in tumour tissues and seldom observed in blood circulation unless in invasive stage [34,35].

We detected DNA methylation of blood samples of only metastatic cancer cases. Therefore blood DNA will not be useful for early detection of bladder cancer by these markers while urine sediment DNA might be more helpful in this regard. However, our results are indicating that blood DNA particularly might be useful for predicting patient survival and tumor progression from noninvasive to invasive stage. This was previously reported by other authors on the different type of cancers [36,37].

Moreover, our findings have provided strong evidence regarding the diagnostic potential of emerging epigenetic markers in bladder cancer patients. These

Table 3
The relationship between *P14ARF* promoter methylation and clinicopathological parameters of bladder cancer blood and tissue samples

Characteristic	Overall		Methylated		UnMethylated		*P* Value	
	blood	tissue	blood	tissue	blood	tissue	blood	tissue
Age ($n = 80$)								
$\leqslant 50$	22		0 (0.0%)	2 (9%)	22 (100%)	20 (91%)	0.006	0.01
> 50	58		8 (13.8%)	16 (27.58%)	50 (86.2%)	42 (72.42%)		
Smoking ($n = 80$)								
Smoker	31		5 (16.1%)	10 (32.2%)	26 (83.9%)	21 (67.8%)	0.2	0.1
Non smoker	49		3 (6.1%)	8 (16.3%)	46 (93.9%)	41 (83.4%)		
Stages ($n = 80$)								
superficial $\leqslant$ T1	63		2 (3.2%)	10 (15.9%)	61 (96.8%)	53 (84.1%)	0.00001	0.0002
invasive $\geqslant$ T2	17		6 (35.3%)	8 (47.1%)	11 (64.7%)	9 (52.9%)		

Table 4
The relationship between *P16INK4a* promoter methylation and clinicopathological parameters of bladder cancer blood and tissue Samples

Characteristic	Overall		Methylated		UnMethylated		*P* Value	
	blood	tissue	blood	tissue	blood	tissue	blood	tissue
Age ($n = 80$)								
$\leqslant 50$	22		3 (13.6%)	9 (41%)	19 (86.4%)	13 (59%)	0.95	0.95
> 50	58		9 (15.5%)	23 (40%)	49 (85.5%)	35 (60%)		
Smoking ($n = 80$)								
Smoker	31		6 (19.4%)	14 (45.2%)	25 (80.68%)	17 (54.8%)	0.57	0.53
Non smoker	49		6 (12.25%)	18 (36.7%)	43 (87.75%)	31 (63.3%)		
Stages ($n = 80$)								
superficial $\leqslant$ T1	63		4 (6.3%)	22 (34.9%)	59 (93.7%)	41 (65.1%)	0.00005	0.01
invasive $\geqslant$ T2	17		8 (47.1%)	10 (58.8%)	9 (52.9%)	7 (41.2%)		

molecular assays may help developing more appropriate therapeutic interventions and management of clinical outcome. Inclusion of the other relevant tumor suppressor genes in the current panel of markers may increase the sensitivity of the assayOn the other hand, it is possible that some bladder tumour may not carry any epigenetic alteration and, thus, should be detected by other approaches, such as mutation analysis, LOH analysis, or detection of homozygous deletions [38–40].

Aging is one of the main risk factors for cancer and the highest cancer rates exist among people over the age of 70 [41]. Epigenetic alterations closely related to the aging process have also been studied already and are proposed to be part of the primary processes contributing to age-related diseases such as cancer [42].

An increase in de novo methylation is an age-related event that occurs in normal tissue and may progress to regional hypermethylation, leading to silencing of specific genes important in the suppression of tumor formation [43]. There are some environmental and mitochondrial factors (intrinsic pathway of apoptosis) which can affect age-related methylation and cancer.

Environmental factors known to contribute to methylation alterations include carcinogen exposures, inflammation, and diet. Several carcinogen exposures such as tobacco, alcohol, arsenic, and asbestos have been associated with methylation-induced gene-inactivation in various human cancers including bladder cancer, head and neck squamous cell carcinoma, and mesothelioma [45–49].

It is therefore reasonable to suggest that various and potentially accumulating exposures throughout life may directly or indirectly lead to methylation alterations and impact disease susceptibility. In the present study, no significant association was found between the risk of tobacco, high risk job, alcohol consumption and P14ARF and P16INK4A hypermethylation. Controversially, Methylation of CpG islands in tobacco- associated cancers occurs in CDKN2A and directly or indirectly is induced by exposure to tobacco smoke in non-small-cell lung carcinoma [50]. Moreover, Wolff et al. (2008) observed that the prevalence of methylation at RUNX3 increased as a function of age at diagnosis and a history of smoking in bladder tumour.

Moreover, we found that the incidence of P14ARF hypermethylation significantly increased in the tissue and blood samples of bladder cancer patients with an increase in age. A study by Brock et al., (2009) has supported our findings and, in addition, they showed that age-related alterations in CpG loci were tissue-dependent. More importantly, they suggested that the

relationship between aging and promoter CpG methylation is complex.

On the other hand, other internal factors which are related to mitochondria and aging such as oxidative damage may have an effect on methylation and age-related cancer. It has been demonstrated that reactive oxygen species may cause oxidative damage and lead to mutations of mtDNA and alterations of the expression of several clusters of genes in aging tissues and senescent cells [53]. Mitochondrial defects have long been suspected to play an important role in the development and progression of cancer [54]. Xie et al., (2007) provided the first evidence regarding involvement of the mtDNA content in regulation of the promoter methylation and an effect on cancer.

Our results support previous and its current that methylation of some genes are tightly correlated in tumors as well as genes which show age-related methylation in bladder cancer [52]. Our data suggest that although environment factors have no effect on methylation, some mitochondrial factors (Intrinsic pathway factors) may play a role and need to be evaluated more.

Organ-specific panels of loci could be developed reflecting global epigenetic events. This would be small enough as a practical tool for routine applications. Further multi-institutional studies are required to validate these markers and their general applicability in the management of patients with urothelial cancer.

9. Conclusion

These results suggest that the CpG island hypermethylation status of the defined panel of genes may be a useful biomarker in patients suffering from bladder cancer.

References

[1] U.S. National Cancer Institute, bladder cancer. Available at: http://www.cancer.gov/bladder accessed November1, 2008.

[2] M. Vaish, A. Mandhani, R.D. Mittal and B. Mittal, Microsatellite instability as prognostic marker in bladder tumors: a clinical significance, *BMC Urology* **5** (2005), 2471–2490.

[3] D. Matthew, Galsky and W. Robert, Bladder Cancer, *Text book of Kidney and Urinary Tract*, 8th Edition. Wolters Kluwer: Lippincott Williams&Wilkins Co., 2007, 777–785.

[4] M.P. Zeegers, R.A. Goldbohm and P.A. van den Brant, A prospective study on active and environmental tobacco smoking and bladder cancer risk, *Cancer Causes Control* **13**(1) (2002), 83–86.

[5] http://www.oncologyradiation.com/, radiation-therapy-bladder-cancer.

[6] D. Sidransky, P. Frost, A. von Eschenbach, R. Oyasu, A.C. Preisinger and B. Vogelstein, Clonal origin of bladder cancer, *N Engl J Med* **326** (1992), 737–740.

[7] L. Mao, D.J. Lee, M.S. Tockman, Y.S. Erozan, F. Askin and D. Sidransky, Microsatellite alterations as clonal markers for the detection of human cancers, *Proc Natl Acad Sci USA* **91** (1994), 9871–9875.

[8] T. Habuchi, R. Takahashi, H. Yamada, Y. Kakehi, T. Sugiyama and O. Yoshida, Metachronous multifocal development of urothelial cancers by intraluminal seeding, *Lancet* **342** (1993), 1087–1088.

[9] I.H. Wong, Y.M. Lo and P.J. Johnson, Epigenetic tumor markers in plasma and serum: biology and applications to molecular diagnosis and disease monitoring, *Ann N Y Acad Sci* **945** (2001), 36–50.

[10] S.B. Baylin and J.G. Herman, DNA hypermethylation in tumorigenesis: epigenetics joins genetics, *Trends Genet*, **16** (2000), 168–174.

[11] P.A. Jones and S.B. Baylin, The fundamental role of epigenetic events in cancer, *Nat Rev Genet* **3** (2002), 415–428.

[12] M. Partha, Das and Rakesh Singal, DNA Methylation and Cancer, *J Clin Oncol* **22** (2004), 4632–4642. by American Society of Clinical Oncology.

[13] P.A. Jones and P.W. Laird, Cancer epigenetics comes of age, *Nat Genet* **21** (1999), 163–167.

[14] M. Esteller, CpG island hypermethylation and tumor suppressor genes: a booming present, a brighter future, *Oncogene* **21** (2002), 5427–5440.

[15] M. Esteller, Relevance of DNA methylation in the management of cancer, *Lancet Oncol* **4** (2003), 351–358.

[16] A. Patel, J.D. Groopman and A. Umar, DNA methylation as a cancerspecific biomarker: from molecules to populations, *Ann N Y Acad Sci* **983** (2003), 286–297.

[17] S.E. Cottrell and P.W. Laird, Sensitive detection of DNA methylation, *Ann N Y Acad Sci* **983** (2003), 120–130.

[18] P.W. Laird, The power and the promise of DNA methylation markers, *Nat Rev Cancer* **3** (2003), 253–266.

[19] J. Hayslip and A. Montero, Tumor suppressor gene methylation in follicular lymphoma: a comprehensive review, *Molecular Cancer* **5** (2006), 44–48.

[20] J.C. Richard, P.W. Laird and R.H. Datar, Promoter Hypermethylation. A New Therapeutic Target Emerges in Urothelial Cancer, *Journal of Clinical Oncology* **23**(13) (2005), 2879–2881.

[21] M.W. Chan, L.W. Chan and N.L. Tang, Frequent hypermethylation of promoter region of RASSF1A in tumor tissues and voided urine of urinary bladder cancer patients, *Int J Cancer* **104** (2003), 611–616.

[22] L.L. Chang, W.T. Yeh and S.Y. Yang, Genetic alterations of p16INK4a and p14ARF genes in human bladder cancer, *J Urol* **170** (2003), 595–600.

[23] Y. Horikawa, K. Sugano and M. Shigyo, Hypermethylation of an E-cadherin (CDH1) promoter region in high grade transitional cell carcinoma of the bladder comprising carcinoma in situ, *J Urol* **169** (2003), 1541–1545.

[24] G. Dominguez, J. Silva and J.M. Garcia, Prevalence of aberrant methylation of *p14ARF* over *p16INK4a* in some human primary tumors, *Mutat Res* **530** (2003), 9–17.

[25] C. Bernal, F. Aguayo, C. Villarroel, M. Vargas, I. Díaz, F.J. Ossandon, E. Santibáñez, M. Palma, E. Aravena, C. Barrientos and A.H. Corvalan, Reprimo as a potential biomarker for early detection in gastric cancer, *Clin Cancer Res* **14**(19) (2008), 6264–6269.

[26] G. Sharma, S. Mirza, C.P. Prasad, A. Srivastava, S.D. Gupta

and R. Ralhan, Promoter hypermethylation of $p16^{INK4A}$, $p14^{ARF}$, *CyclinD2* and *Slit2* in serum and tumor DNA from breast cancer patients, *Life Science* **80** (2007), 1873–1881.
[27] C.S. Chim, Y.L. Kwong and R. Liang, Gene hypermethylation in multiple myeloma: lessons from a cancer pathway approach, *Clin Lymphoma Myeloma* **8**(6) (2008), 331–339.
[28] R.T. Williams and C.J. Sherr, The INK4-ARF (CDKN2A/B) Locus in Hematopoiesis and BCR-ABL-induced Leukemias, Cold Spring Harb Symp Quant Biol. 2008 Nov 26. [Epub ahead of print].
[29] S.J. Gallagher, J.F. Thompson, J. Indsto, L.L. Scurr, M. Lett, B.F. Gao, R. Dunleavey, G.J. Mann, R.F. Kefford and H. Rizos, p16INK4a expression and absence of activated B-RAF are independent predictors of chemosensitivity in melanoma tumors, *Neoplasia* **10**(11) (2008), 1231–1239.
[30] C. Fan, L. He, A. Kapoor, A. Gillis, A.P. Rybak, J.C. Cutz and D. Tang, Bmi1 promotes prostate tumorigenesis via inhibiting p16(INK4A) and p14(ARF) expression, *Biochim Biophys Acta* **1782**(11) (2008), 642–648.
[31] J.G. Herman, J.R. Graff, S. Myöhänen, B.D. Nelkin and S.B. Baylin, Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands, *PNAS* **93** (1996), 9821–9826.
[32] E.P. Xing, Y. Nie, Y. Song, G.Y. Yang, Y.C. Cai, L.D. Wang and C.S. Yang, Mechanisms of inactivation of p14ARF, p15INK4b, and p16INK4a genes in human esophageal squamous cell carcinoma, *Clin Cancer Res* **5**(10) (1999), 2704–2713.
[33] J.F. Viallard, F. Lacombe, F. Belloc, J.L. Pellegrin and J. Reiffers, Molecular mechanisms controlling the cell cycle: fundamental aspects and implications for oncology, *Cancer Radiother* **5**(2) (2001), 109–129.
[34] K. Yamashita, S. Upadhyay and M. Osada, Pharmacologic unmasking of epigenetically silenced tumor suppressor genes in esophageal squamous cell carcinoma, *Cancer Cell* **2** (2002), 485–495.
[35] C. Jeronimo, R. Henrique and M.O. Hoque, A quantitative promoter methylation profile of prostate cancer, *Clin Cancer Res* **10** (2004), 8472–8478.
[36] D.R. Yates, I. Rehman, M.F. Abbod, M. Meuth, S.S. Cross, D.A. Linkens, F.C. Hamdy and J.W.F. Catto, Promoter Hypermethylation Identifies Progression Risk in Bladder, *Cancer-Clinical Cancer Research* **13** (2007), 2046.
[37] L. Shen, H. Kantarjian, Y. Guo, E. Lin, J. Shan, X. Huang, D. Berry, S. Ahmed, W. Zhu, Sh. Pierce, Y. Kondo, Y. Oki, J.Jelinek, H. Saba, E. Estey and J.J. Issa, DNA Methylation Predicts Survival and Response to Therapy in Patients With Myelodysplastic Syndromes, *Journal of Clinical Oncology* **28**(4) (2010), 605–613.
[38] L. Mao, M.P. Schoenberg, M. Scicchitano, Y.S. Erozan, A. Merlo and D. Schwab, Molecular detection of primary bladder cancer by microsatellite analysis, *Science* **271** (1996), 659–662.
[39] G. Steiner, M.P. Schoenberg, J.F. Linn, L. Mao and D. Sidransky, Detection of bladder cancer recurrence by microsatellite analysis of urine, *Nat Med* **3** (1997), 621–624.
[40] P. Cairns, T.J. Polascik, Y. Eby, K. Tokino, J. Califano and A. Merlo, Frequency of homozygous deletion at p16/CDKN2 in primary human tumours, *Nat Genet* **11** (1995),210–212.
[41] M. Esteller, M.F. Fraga, M.F. Paz, E. Campo, D. Colomer, F.J. Novo, M.J. Calasanz, O. Galm, M. Guo, J. Benitez and J.G. Herman, Cancer epigenetics and methylation, *Science* **297** (2002), 1807–1808.
[42] M.D. Shahbazian and H.Y. Zoghbi, Rett syndrome and MeCP2: linking epigenetics and neuronal function, *Am J Hum Genet* **71** (2002), 1259–1272.
[43] N. Lopatina, J.F. Haskell, L.G. Andrews, J.C. Poole, S.Saldanha and T.O. Tollefsbol, Differential maintenance and de novo methylating activity by three DNA methyltransferases in aging and immortalized fibroblasts, *J Cell Biochem* **84** (2002), 324–334.
[44] B.C. Christensen, J.J. Godleski, C.J. Marsit, E.A. Houseman and C.Y. Lopez-Fagundo. Asbestos exposure predicts cell cycle control gene promoter methylation in pleural mesothelioma, *Carcinogenesis* **29** (2008), 1555–1559.
[45] C.J. Marsit, E.A. Houseman, A.R. Schned, M.R. Karagas and K.T. Kelsey, Promoter hypermethylation is associated with current smoking, age, gender and survival in bladder cancer, *Carcinogenesis* **28** (2007), 1745–1751.
[46] C.J. Marsit, M.D. McClean, C.S. Furniss and K.T. Kelsey, Epigenetic inactivation of the SFRP genes is associated with drinking, smoking and HPV in head and neck squamous cell carcinoma, *Int J Cancer* **119** (2006), 1761–1766.
[47] S. Toyooka, R. Maruyama, K.O. Toyooka, D. McLerran and Z. Feng, Smoke exposure, histologic type and geography-related differences in the methylation profiles of non-small cell lung cancer, *Int J Cancer* **103** (2003), 153–160.
[48] C.M. Lyon, D.M. Klinge, K.C. Liechty, F.D. Gentry and T.H. March, Radiation-induced lung adenocarcinoma is associated with increased frequency of genes inactivated by promoter hypermethylation, *Radiat Res* **168** (2007), 409–414.
[49] B.C. Christensen, E.A. Houseman, J.J. Godleski, C.J. Marsit, J.L. Longacker, Epigenetic profiles distinguish pleural mesothelioma from normal pleura and predict lung asbestos burden and clinical outcome, *Cancer Res*, 2008.
[50] D.H. Kim, H.H. Nelson, J.K. Wiencke, D.C. Christiani, J.C. Wain, E.J. Mark and K.T. Kelsey, Promoter hypermethylation of DAP-kinase: association with advanced stage in non-small cell lung cancer, *Oncogene*, 2001, in press.
[51] E.M. Wolff, G. Liang, C.C. Cortez, Y.C. Tsai, J.E. Castelao, V.K. Cortessis, D.D. Tsao-Wei, S. Groshen and P.A. Jones, RUNX3 Methylation Reveals that Bladder Tumors Are Older in Patients with a History of Smoking, *Cancer Research* **68** (2008), 6208–6214.
[52] C. Ch. Brock, E.A. Houseman, J.M. Carmen, Sh. Zheng, R.W. Margaret, J.L. Wiemels, H.H. Nelson, M.R. Karagas, J.F. Padbury, R. Bueno, D.J. Sugarbaker, R. Yeh, J.K. Wiencke and K.T. Kelsey, Aging and Environmental Exposures Alter Tissue-Specific DNA Methylation Dependent upon CpG Island Context, *PLoS Genet* **5**(8) (2009), e1000602.
[53] Y. Wei, S. Wu, Y. Ma and H. Lee, Respiratory Function Decline and DNA Mutation in Mitochondria, Oxidative Stress and Altered Gene Expression during Aging, *Chang Gung Med J* **32** (2009), 113–132.
[54] W.C. Copeland, J.T. Wachsman, F.M. Johnson and J.S. Penta, Mitochondrial DNA alterations in cancer, *Cancer Invest* **20** (2002), 557–569.
[55] C. Xie, A. Naito, T. Mizumachi, T.T. Evans, M.G. Douglas, C.A. Cooney, C. Fan and M. Higuchi, Mitochondrial regulation of Cancer Associated Nuclear DNA Methylation, *Biochem Biophys Res Commun* **364**(3) (2007), 656–661.