Biomarkers, 2010; 15(2): 167–174

informa
healthcare

ORIGINAL ARTICLE

Hypermethylation-mediated partial transcriptional silencing of DAP-kinase gene in bladder cancer

Ranbir Chander Sobti[1], Kianoosh MalekZadeh[1], Mohsen Nikbakht[1], Irandokht Asadi Sadeghi[2], Mohammad Shekari[1], and Shrawan Kumar Singh[3]

[1]*Department of Biotechnology, Panjab University, Chandigarh, India,* [2]*Medical Faculty, Zabol Medical University, Zabol, Iran and* [3]*Advanced Urology Department, Post Graduate Institute of Medical Education and Research (PGIMER), Chandigarh, India*

Abstract

Death-associated protein kinase (DAP-kinase) is a novel serine/threonine kinase whose expression is required for interferon-γ-induced apoptosis. This study evaluated the methylation pattern and its impact on the expression of the DAP-kinase gene in transitional cell carcinoma of the bladder as hypermethylation is one of the earliest and most frequent alterations leading to cancer. The frequency of hypermethylation of the gene promoter was 37.8%. On correlation with clinicopathological features, methylation was seen mostly in superficial tumours in the group aged > 60 years (42.9 vs 33.3% of those ≤ 60 years) and in smokers (48.1 vs 27.4% of non-smokers). The increased risk of bladder cancer was 6.70-fold (95% confidence interval (CI) 2.09–23.87; $p = 0.000$) in those carrying methylated DAP-kinase and it was elevated in patients who smoked (odds ratio 7.87; 95% CI 1.50–54.96; $p = 0.007$). This study demonstrated that methylation in the gene promoter on its own could significantly decrease the mRNA expression level of DAP-kinase by 27.68%. Interestingly, patients within the group aged > 60 years and with a smoking habit showed increased downregulation of mRNA compared with non-smokers of this age group (similar pattern of methylation). Hypermethylation can decrease the expression of DAP-kinase and may be one of the reasons for conversion of normal cells to malignant cells, as the frequency of methylation at the early stage (superficial) of tumours was elevated. Methylation of DAP-kinase can be considered as one of the prognosis indicators for progression and development of bladder cancer.

Keywords: *DAP-kinase; Hypermethylation; bladder cancer*

Introduction

Cancer of the urinary bladder is estimated to be the seventh most prevalent type of cancer worldwide, accounting for approximately 3.2% of the international cancer burden. The 2004 World Health report attributes an estimated 125 000 deaths in males and 54 000 deaths in females worldwide as a result of urothelial carcinoma. In the USA, urothelial cancer (UC) is the fourth leading cause of new cancers in males, and an estimated 61 420 new cases were detected in 2006, with 13 060 succumbing to the disease. The 5-year survival rates for localized cancers and distant metastasis were 94 and 6%, respectively (Mitra et al. 2006). According to the US Census Bureau International database, an estimated 221 237 new cases of bladder cancer have been diagnosed. Considering the size of the Indian population (1 065 070 607), its rank in the world incidence is very low (<5 per 100 000 population) (Cancer Research UK; http://info.cancerresearchuk.org/cancerstats/types/bladder/?a = 5441).

Epigenetics describes a stable alteration in the potential gene expression that takes place during development and cell proliferation, without any change in the gene sequence (Herman et al. 1996). Hypermethylation of the CpG islands of the gene promoter is one of the earliest and most frequent alterations leading to cancer. It is an important epigenetic mechanism

Address for Correspondence: Kianoosh MalekZadeh, Department of Biotechnology, Panjab University, Chandigarh, India. E-mail: keyanoosh@gmail.com

(Received 22 June 2009; revised 06 October 2009; accepted 07 October 2009)

ISSN 1354-750X print/ISSN 1366-5804 online © 2010 Informa UK Ltd
DOI: 10.3109/13547500903395124

for gene silencing, which may confer tumour cells with a growth advantage (Baylin & Herman 2000).

One of the hallmarks of cancer is an imbalance between cell growth and death. Whereas normal cells undergo apoptosis after specific regulatory signals, transformed cells can lose this response and survive, accumulating genetic and phenotypic defects leading to tumour progression. In particular, DNA damage-induced apoptosis in response to genotoxic drug treatment or ionizing radiation may be diminished. Critical genes involved in this response include p53, Bcl-2 and Bax (Katzenellenbogen et al. 1999).

Death-associated protein kinase (DAP-kinase) is a 160-kDa serine/threonine, microfilament-bound kinase shown to be involved in interferon-γ-induced apoptosis (Cohen et al. 1997); it is involved in the p53-dependent apoptosis pathway (Raveh et al., 2001). It prevents the translocation of extracellular signal-regulated kinase (ERK) protein from the cytoplasm into the nucleus, thereby inhibiting signal transduction. The gene (DAP-kinase) may be biologically important in normal cell differentiation and selection in the immune system (Mitra et al. 2006). Overexpression of DAP-kinase induces programmed cell death (Katzenellenbogen et al. 1999; Cohen et al. 1997). Several B-cell lymphoma cell lines have decreased or absent expression of DAP-kinase RNA or protein. Some of these cell lines are able to re-express DAP-kinase after treatment with 5-aza-2'-deoxycytidine, a DNA-demethylating drug (Kissil et al. 1997). This finding suggests that abnormal loss of DAP-kinase expression could be associated with aberrant promoter region methylation, serving as an alternative to the genetic loss of a tumour-suppressor gene function by deletion or mutation (Katzenellenbogen et al. 1999, Baylin et al. 1998).

Non-invasive and early cancer detection methods can be developed by study of epigenetic changes. A wide range of methylation levels have been reported; therefore, in this study, we tried to study the methylation pattern of DAP-kinase in bladder cancer in a north Indian population (according to the best of our knowledge, this is the first report in an Indian population) and also to investigate the effect of this epigenetic event on silencing or loss of expression level of DAP-kinase together with some clinicopathological factors.

Material and methods

Study subjects and specimens

A total of 103 fresh tissue samples were collected between July 2004 and March 2007, with informed consent, from patients with bladder cancer (totally transitional cell carcinomas) in the Advanced Urology Department (AUC), Post Graduate Institute of Medical Education and Research (PGIMER), Chandigarh, India, immediately after transurethral resection of bladder tumour (TURBT) or radical cystectomy. None of the patients had received radio- and/or chemotherapy. Collected samples were then snap-frozen in liquid nitrogen and stored at -80°C until processing. All the specimens were examined histologically by the histopathology department of PGIMER to confirm (1) the diagnosis, (2) the presence of a tumour and to evaluate the percentage of tumour cells in each sample, and (3) the pathological staging. All the samples showed at least 75% tumour cells. Histopathological staging and grading followed the TNM classification of the International Union Against Cancer (IUAC). Briefly, superficial tumours were classified as T1 or Ta, while those with deep muscular invasion were assigned as T2 or T3.

A total of 48 bladder tissue samples from patients who were confirmed negative histologically of any kind of malignancy by the histopathology department were used as a control group. The clinical and pathological characteristics as well as detailed data such as age, gender, history and smoking status of all the patients and healthy controls were obtained from an interviewer-administered questionnaire and are summarized in Table 1. People with pack-years more than 20 has been classified as continues smokers, respectively. In this study, we considered 20 cigarettes calculated as one pack.

DNA isolation

DNA was extracted from frozen tissue samples using SDS–proteinase K treatment and phenol–chloroform extraction and ethanol precipitation, which is a traditional standard procedure.

Sodium bisulfite modification of DNA

Bisulfite modification of genomic DNA was performed according to the technique of Herman et al. (1996). Briefly, 2 mg of genomic DNA was denatured with NaOH (final concentration 0.2 M) for 10 min at 37°C, and 30 ml hydroquinone (10 mM) (Sigma-Aldrich, Bangalore, India) and 520 ml sodium bisulfite (3 M) (SRL, New Delhi, India) at pH 5.0 were added and incubated at 50°C for 16 h. Afterwards, modified DNA was purified using Wizard DNA purification resin (Promega, Chennai, India) and eluted into 50 µl of water and then followed by desulfonation in 0.3 N NaOH at 25°C for 5 min. Desulfonated DNA was then precipitated with ammonium acetate and ethanol, washed with 70%

ethanol and resuspended in 20 µl H_2O. Treatment of genomic DNA with sodium bisulfite converted unmethylated cytosines (but not methylated cytosines) to uracil, which were then converted to thymidine during the subsequent polymerase chain reaction (PCR) step, giving sequence differences between methylated and unmethylated DNA.

Mutagenically separated PCR

Mutagenically separated (MS)-PCR was used to examine the methylation status of the CpG island regions of DAP-kinase. The PCR reaction mixture (50 µl) contained 2 µl of the bisulfite-treated DNA, 0.2 mmol l^{-1} of dNTP (each at a concentration of 10 mM) (Fermentas Life Science, New Delhi, India), 10 pmol of each primers (synthesized by Sigma), 5 µl of BSA (10 mg ml^{-1}) (Sigma), 10x PCR buffer (750 mM Tris–HCl (pH 8.8), 200 mM $(NH_4)SO_4$, 0.1% Tween 20), 2.5 mmol l^{-1} $MgCl_2$ and 1 unit of thermostable HotStart Taq DNA polymerase (Qiagen, Hilden, Germany).

The PCR consisted of an initial denaturation for 10 min at 95°C, followed by 40 cycles with an annealing temperature of 64°C. Primer sequences for the unmethylated reaction were 5′-GGA GGA TAG TTG GAT TGAGTTAAT GTT-3′ (sense) and 5′-CAA ATC CCT CCC AAA CAC CAA-3′ (antisense). Primer sequences for the methylated reaction were 5′-GGA TAG TCG GAT CGA GTT AAC GTC-3′ (sense) and 5′-CCC TCC CAA ACG CCG A-3′ (antisense) (Gonzalez-Gomez et al. 2003). The MS-PCR products were visualized under ultraviolet (UV) transillumination after an electrophoretic separation on a 2.5% agarose gel. The size of the MS-PCR product for the unmethylated reaction was 106-bp, whereas that of methylated reaction, it was 98 bp (Figure 1).

RNA isolation

In order to compare the level of RNA expression between the methylated and unmethylated gene in bladder tumours and to determine the effect of hypermethylation, some of the samples were dipped in 0.5 ml RNAlater (Ambion, Haryana, India) after surgery and stored at -80°C for further processing. Total tissue RNA was extracted using TRI reagent (Sigma-Aldrich), in accordance with the manufacturer's instructions. The quality of the RNA was determined by electrophoresis through denaturing agarose gels stained with ethidium bromide; the 18S and 28S RNA bands were visualized under UV light. The yield was quantified spectrophotometrically at 260 and 280 nm.

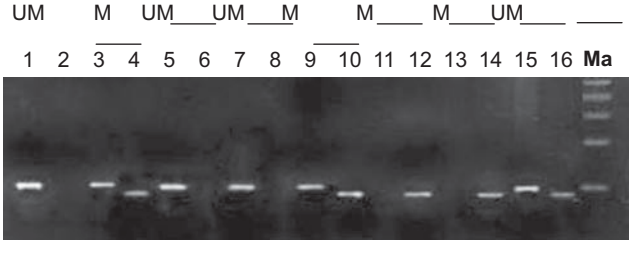

Figure 1. Methylation study of the DAP-kinase gene in bladder cancer by mutagenically separated polymerase chain reaction analysis. Ma, 100 bp marker; M, methylated; UM, unmethylated; lanes 1, 3, 5, 7, 9, 11, 13, 15, result of unmethylated primer application; lanes 2, 4, 6, 8, 10, 12, 14, 16, result of methylated primer application.

Semiquantitative reverse transcriptase-PCR

For the synthesis of first-strand cDNA, 1 µl (0.2 µg $µl^{-1}$) random hexamer primers were added to 1.5 µg of total cellular RNA in a final volume of 12 µl, and following the instructions of RevertAid first-strand cDNA synthesis kit (Fermentas Life Science), 1 µl M-ULV reverse transcriptase was added to the mixture and incubated for 1 h at 42°C with a final step at 70°C for 10 min. Two microlitres of this cDNA was amplified in a total volume of 25 µl PCR reaction mixture containing 5 µl 10x reaction buffer (100 mM Tris–HCl (pH 8.8), 500 mM KCl, 0.8% Nonidet P40), 0.2 mM of each dNTP (Fermentas Life Science), 5 µl of BSA (10 mg ml^{-1}) (Sigma-Aldrich), 2.5 mM $MgCl_2$ and 1 unit of Taq DNA polymerase (Fermentas Life Science) and using 10 pmol of appropriate primers (Sigma-Aldrich).

The sequences of the primers for DAP-kinase were 5′-GAT AGA AAT GTC CCC AAA CCT CG-3′ and 5′-TCT TCT TGG ATC CTT GAC CAG AA-3′, which amplified a 343-bp product (Raveh et al. 2001) and for β-actin were 5′-GTG GGG CGC CCC AGG CAC CA-3′ and 5′-CTC CTT AAT GTC ACG CAC GAT TTC-3′ which amplified a 548 bp product (Mishina et al. 1996). The annealing temperatures for DAP-kinase and β-actin were 58°C and 55°C, respectively, for 45 s and 30 cycles. The PCR product was run on 2% agarose gel and then stained with ethidium bromide and the optical density was measured by Scion-image software. The amount of mRNA expression was calculated by grading a ratio between the densito-metry results of DAP-kinase and β-actin (Figure 2).

Statistical analysis

The obtained data were analysed by using Student's *t*-test and the Mann–Whitney test to compare the expression in patients and controls. The χ^2 test, Fisher's exact test and multivariate logistic regression with adjustment for age were used to find out any difference between cases and controls according to methylation, stage of tumour and smoking status. The reported odds ratio (OR) can be interpreted as age-adjusted estimates

of the relative risk (RR) of developing bladder cancer with methylation of the studied genes. Statistical analysis was performed using SPSS, version 11.5 and Epical, version 3.2.

Results

The clinical characteristics and demographic features of the studied patients are shown in Table 1. The cases and controls were well matched on age, race and sex. The mean age (± SD) of the cases was 60.96 ± 10.84 and, that of controls was 61.00 ± 9.08 and there was no significant difference ($p = 0.983$). The number of male and female subjects in the case and control groups was very similar (male 88.3 (87.5%), female 11.7 (12.5%)). The majority of the patients with bladder cancer were in the age group 50–70 years at diagnosis, and were at stages T1 (38.8%) and T2 (33.0%) of the disease, and histologically were grade 2 (61.2%). The frequency match on smoking status between patients and controls was 49.5 and 33.3% for smokers, 50.5 and 66.7% for non-smokers, respectively. Moreover, the cancer cases had a higher mean value of pack-years (± SD) (46.69 ± 22.08) than the controls (38.19 ± 13.10) ($p = 0.149$).

Table 2 shows the frequency of methylation of DAP-kinase in the patients and controls: 37.8% of the patients and 8.3% of controls showed methylated DAP-kinase. The frequency of methylation of DAP-kinase showed a statistically significant difference between cases and controls ($p = 0.000$). The methylation of the CpG island in the DAP-kinase promoter increased the risk of bladder cancer by 6.70-fold (95% confidence interval (CI) 2.09–23.87).

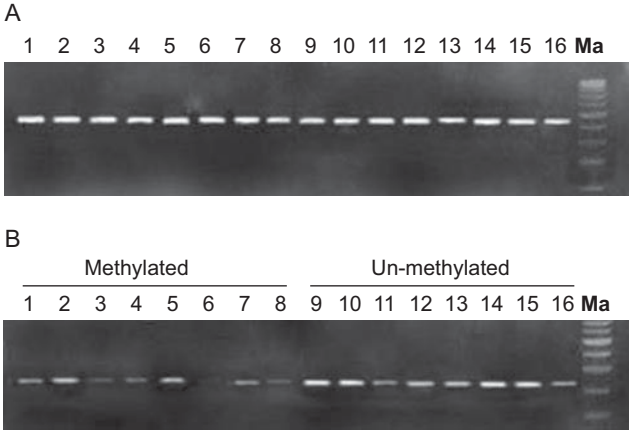

Figure 2. The effect of methylation on the expression of the DAP-kinase gene. (A) Expression of β-actin used as a housekeeping gene (548 bp). (B) Expression of the DAP-kinase gene (343 bp); lanes 1–8, expression of DAP-kinase in a patient with methylated promoter of the DAP-kinase gene; lanes 9–16, with unmethylated promoter.

The impact of age, tumour stage and smoking habit with methylation of DAP-kinase and development of bladder cancer is summarized in Table 2. The percentage of methylated DAP-kinase in the tissue of cases aged ≤ 60 years was 33.3% (18 out of 54) and 42.9% (21 out of 49) in the cases aged > 60 years. The methylation of DAP-kinase was significantly different between cases and controls in both age groups ($p = 0.006$ for age ≤ 60 years and $p = 0.002$ for age > 60).

DAP-kinase promoter was methylated in 40.4% (23/57) of the superficial (stages Ta + T1) and in 37.0% (17/46) of the muscle-invasive (stage ≥ pT2) tumours. Methylation of DAP-kinase significantly increased the risk of developing bladder cancer (OR 8.00, 95% CI 2.32–30.31 and RR 2.00 in superficial tumours; OR 5.32, 95% CI 0.97–35.59 and RR 1.91 in muscle-invasive tumours).

Methylation was seen in 48.1% (25 out of 52) of patients who were smokers, but was 27.4% (14/51) in non-smokers. Methylation of the DAP-kinase promoter significantly increased the risk for bladder cancer in smokers (OR 7.87, 95% CI 1.50–54.96, RR 1.51, $p = 0.007$) as well as non-smokers (OR 5.11, 95% CI 0.97–35.59, $p = 0.028$) compared with the healthy controls.

In order to investigate the effect of smoking on hypermethylation, patients who were smokers were compared with non-smokers. Smoking significantly increased the chance of hypermethylation in the promoter of DAP-kinase by 2.45-fold (95% CI 1.00–6.06, $p = 0.031$).

The effect of methylation on the mRNA expression of DAP-kinase is shown in Table 3 and Figure 3. The RNA expression was significantly reduced in patients

Table 1. Clinical characteristics and demographic features of the study population.

Variables	Patients ($n = 103$) n (%)	Controls ($n = 48$) n (%)
Age (years), mean (± SD), (min–max)	60.96 (± 10.84), (34–85)	61.00 (± 9.08), (44–82)
Age ≤ 60 years	54 (52.4)	23 (47.9)
Age > 60 years	49 (47.6)	25 (52.1)
Sex		
Male	91 (88.3)	42 (87.5)
Female	12 (11.7)	6 (12.5)
Stage (TNM)		
Superficial stages (≤ pT1)	57 (55.3)	
Higher stages (≥ pT2)	46 (44.7)	
Grade		
G1	19 (18.4)	
GII	63 (61.2)	
GIII	21 (20.4)	
Smoking status		
Never smoked	52 (50.5)	19 (39.5)
Smoker	51 (49.5)	29 (60.5)

with methylated DAP-kinase promoter ($p = 0.000$) in comparison with patients with unmethylated DAP-kinase.

In order to study the effect of promoter hypermethylation on the expression of DAPK in bladder cancer, the level of mRNA expression was determined and compared in methylated and unmethylated samples of only patients

The hypermethylation in the promoter resulted in downregulation of RNA expression by about 27.68%. A significant difference in the expression due to methylation in the promoter of DAP-kinase was observed in both age groups ($p = 0.011$ for age > 60 and $p = 0.000$ for age ≤ 60 years). The percentage down-regulation of mRNA expression in the age group > 60 years was 27.8%, but 18.6% in the age group ≤ 60 years.

Methylation of the DAP-kinase promoter significantly reduced the expression both at the superficial (Ta + T1) and muscle-invasive stages of tumours (≥ pT2). The percentage downregulation for superficial tumours and muscle-invasive tumours was 20.9% and 17.13%, respectively.

The impact of smoking on decreasing the RNA expression between methylated and unmethylated

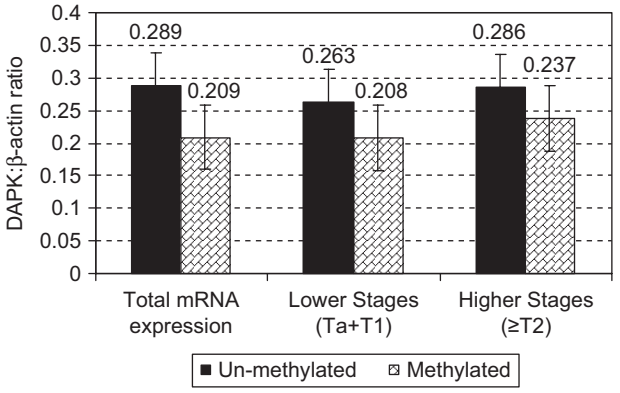

Figure 3. mRNA expression level of DAP-kinase under the effect of promoter hypermethylation.

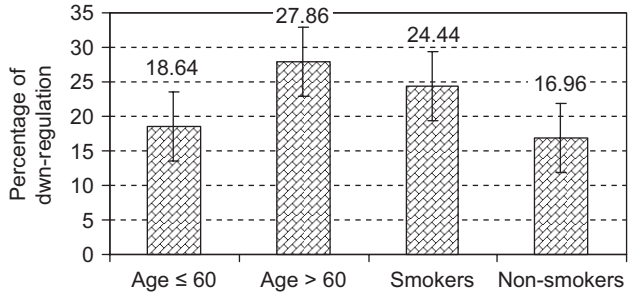

Figure 4. The percentage downregulation of mRNA expression as a result of hypermethylation.

Table 2. The frequency and percentage of methylation of DAP-kinase and clinical–pathological and smoking habit of patients with bladder cancer compared with healthy controls.

Methylation	Patients *n* (%)	Controls *n* (%)	OR (95% CI)	RR (95% CI)	*p*-Value
Total methylation	39 (37.8)	4 (8.3)	6.70 (2.09–23.87)	1.53 (1.27–1.84)	0.000
Age group					
≤ 60 years	18 (33.3)	2 (8.6)	5.50 (1.06–38.0)	1.45 (1.33–1.86)	0.006
> 60 years	21 (42.8)	2 (8.0)	8.63 (1.67–2.20)	1.66 (1.26–2.20)	0.002
Stage of tumour					
Early (Ta+T1)	24 (42.1)	-	8.00 (2.32–30.31)	2.00 (1.48–2.70)	0.000
Higher (≥T2)	15 (26.3)	-	5.32 (1.45–21.19)	1.91 (1.34–2.73)	0.007
Smoking status					
Smoker	25 (48.1)	2 (10.5)	7.87 (1.50–54.96)	1.51 (1.17–1.95)	0.007
Never smoked	14 (27.4)	2 (6.9)	5.11 (0.97–35.59)	1.51 (1.14–2.00)	0.028

OR, odds ratio; CI, confidence interval; RR, relative risk. Percentages calculated for each group, for example, number of patients aged > 60 years was 54.

Table 3. The amount of RNA expression in methylated and unmethylated DAP-kinase in patients and the impact of clinicopathological characteristics and smoking on the percentage downregulation of RNA expression.

	Unmethylated	Methylated	Downregulation (%)	*p*-Value
Total mRNA expression	0.289 (± 0.043)	0.209 (± 0.041)	27.68	0.000
Age group				
≤ 60 years	0.279 (± 0.046)	0.227 (± 0.037)	18.64	0.011
> 60 years	0.280 (± 0.044)	0.202 (± 0.041)	27.86	0.000
Stages				
Early stage (Ta+T1)	0.263 (± 0.030)	0.208 (± 0.042)	20.91	0.000
Higher stage (≥T2)	0.286 (± 0.049)	0.237 (± 0.017)	17.13	0.007
Smoking status				
Smokers	0.276 (± 0.047)	0.201 (± 0.040)	27.17	0.000
Non-smokers	0.289 (± 0.042)	0.240 (± 0.032)	16.96	0.003

DAP-kinase was significant in both smokers and non-smokers. The percentage downregulation of mRNA expression caused by methylation in patients who were smokers was 27.17% ($p = 0.000$), whereas it was 16.96% ($p = 0.003$) in non-smokers (Table 3). Figure 4 shows that the downregulation caused by hypermethylation was higher in the group aged > 60 years and in patients who smoke.

Discussion

The silencing of genes by promoter hypermethylation is common in human cancers. Although the mechanisms of the epigenetic changes are not clearly understood, the list of aberrant cases of methylation of genes in cancer is rapidly growing. Nevertheless, a certain kind of tissue-specific methylated gene has been demonstrated. For example, a high frequency of methylation of *GSTP1* has been found in prostate cancer (Esteller et al. 1998). Epigenetic changes have also been detected in DNA from plasma/serum, urine and sputum, indicating that a non-invasive and early cancer detection method can be developed (Chan et al. 2002).

Methylation was demonstrated to be the mechanism of loss of expression of DAP-kinase in bladder cancer cells and other cancer cells (Katzenellenbogen et al. 1999, Kissil et al. 1997). A decrease in expression of this gene imparts resistance to interferon-γ-induced apoptosis in cells (Cohen et al. 1997, Deiss et al. 1995) and a link between the loss of DAP-kinase expression and cellular apoptosis may facilitate metastasis in an experimental system (Inbal et al. 1997).

This study addressed the question of whether hypermethylation in the DAP-kinase gene promoter has a significant association with bladder cancer, and also whether this hypermethylation in the promoter has any effect on gene expression. The correlation of these methylation changes and molecular abnormalities with clinicopathological characteristics in a large number of patients with bladder cancer was also evaluated.

In this study, a high frequency of methylation in the DAP-kinase promoter of patients with bladder cancer was observed. DAP-kinase shows variable methylation levels across different studies. Some reports have demonstrated a low frequency of methylation of DAP-kinase in bladder cancer (Esteller et al. 2001, Maruyama et al. 2001, Catto et al. 2005, Yates et al. 2006), but a high frequency of methylation in the DAP-kinase promoter (22%, 58.2% and 74%) has also been reported by other workers (Friedrich et al. 2004, Chan et al. 2002, Christoph et al. 2006). Our observation (37.8%) is in agreement with the latter group. This variability of methylation levels reported for DAP-kinase in bladder cancer is perhaps due to the methods used, such as the sequence of primers or qualification of bisulfite modification of genomic DNA; ethnic and geographical factors and the differences in sample size may also account for the differences in frequency of DAP-kinase methylation.

Bladder cancer is more common in men than women with a worldwide male: female ratio of 10:3 (IARC 2002). In this study, around 12% of samples were from female subjects. No methylated DAP-kinase promoter was observed in female individuals of either the patient or control groups. The low incidence of bladder cancer among women has not enabled a conclusion to be reached regarding the effect of gender on methylation.

Significant differences of methylation of DAP-kinase between patients and controls in both age groups ≤ 60 and > 60 years were observed. Therefore, it can be deduced that methylation of DAP-kinase is independent of age. However, the frequency of methylation (42.9 vs 33.3%), and increased risk of bladder cancer (OR 8.63 vs 5.50) was higher in the group aged > 60 years.

The percentage of smokers among patients with a methylated DAP-kinase promoter was higher compared with non-smokers (48.1 vs 27.4%). In spite of hypermethylation of DAP-kinase on its own increasing the risk of bladder cancer in both smokers and non-smokers, interestingly, it was found that the risk of hypermethylation was elevated 2.5-fold by smoking. To our knowledge, the correlation of smoking and hypermethylation of DAP-kinase has not been reported before.

In order to determine the effect of methylation of DAP-kinase on its mRNA expression – whether it can lead to partial or total silencing of DAP-kinase – the mRNA expression between patients with methylated promoter and patients with unmethylated promoter was compared. It was found that hypermethylation by itself was able to decrease mRNA expression of DAP-kinase (as in tumour suppressor genes) by around 27.5% in bladder cancer. The data on downregulation confirmed the methylation pattern. Therefore, it is concluded that hypermethylation can decrease the expression of DAP-kinase and can be one of the reasons for the conversion of normal cells to malignant cells, as the frequency of methylation at the earlier stages (superficial) of tumours was high.

Other studies also support the fact that the frequency of methylation in the DAP-kinase promoter in superficial tumours and in the initial stages of disease is greater than in muscle-invasive tumours (42.1 vs 32.6%). Christoph et al. (2006) reported a higher level of methylation in DAP-kinase promoter in superficial

tumours than in tumours ≥ pT2, and the extent of methylation in early-stage and advanced-stage tumours varied. In this follow-up study, the DAP-kinase methylation level was elevated in tumour tissue compared with normal urothelial tissue, where the median methylation level was less frequent and quite low (< 5%).

In order to study the effect of promoter hypermethylation on the expression of DAP-kinase in bladder cancer, the level of mRNA expression was determined and compared in methylated and unmethylated samples of only patients.

One mechanism by which methylation can be increased is by upregulation of the expression of DNA methyltransferase-1 (DNMT1), which occurs in cells with loss of retinoblastoma tumour suppressor protein (Rb) function (Merlo et al. 1995). This might explain how with increasing age and the exposure of people to the carcinogens existing in tobacco, the time and dose of exposure in urine will increase the risk of mutation in the *Rb* gene (a key gene in the cell cycle) and, consequently, the probability of affecting superficial tumours, as it has been observed that methylation of DAP-kinase (as in tumour suppressor genes) significantly occurs in the early stage of bladder cancer.

In conclusion, a distinct methylation pattern of the DAP-kinase gene in patients with bladder cancer has been demonstrated because methylation of DAP-kinase promoter as seen in proapoptotic genes is a common event in carcinogenesis. Epigenetic inactivation of proapoptotic genes may prevent the induction of apoptosis, even in cancer cells that express functional p53, which might explain the more aggressive behaviour of tumours with higher levels of promoter methylation (Christoph et al. 2006). These findings may have potential diagnostic and therapeutic implications as a potential non-invasive diagnostic and monitoring tool and deserve further investigation. Classifying tumours based on DNA methylation may provide improved clinical estimates of more aggressive phenotypes and help to identify patients in need of more aggressive treatment strategies. On the other hand, patients with a less malignant phenotype could benefit from extension of cystoscopy intervals in non-muscle-invasive tumour disease. Moreover, the technical ability to demethylate these proapoptotic genes using orally administered agents makes epigenetic investigation a promising field for both diagnostic and therapeutic targeting.

Acknowledgements

The authors wish to thank all the staff and patients who took part in the study. We are also thankful to PGIMER, Chandigarh for providing the clinical samples. We thank the University Grants Commission (U.G.C), Government of India for funding.

***Declaration of interest*:** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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