Effect of DAPK methylation and Bcl2 over expression on risk of recurrence in Transitional Cell Carcinoma of bladder

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Although, several factors dictate bladder cancer progression and patient survival, mechanisms for bladder carcinogenesis and the development of bladder cancer remain unclear. Aberrant methylation of CpG islands and deregulation of apoptosis, which are found in early stages of carcinogenesis are one of the specifications of human cancers. We aimed to investigate expression of the Bcl2 and methylation status of DAPK, in determining the risk for bladder cancer recurrence. DAPK promoter hypermethylation was analyzed in 80 histologically confirmed incidents of Transitional Cell Carcinoma (TCC) of the bladder specimens and 80 healthy samples by MS-PCR. DAPK methylation was found in 36.25% of tissue samples of TCC. The incidence of DAPK hypermethylation showed significant association with an increase in age ($p = 0.01$), recurrence ($p = 0.0001$), and smoking status ($p = 0.03$) but no association was found with other clinicopathological parameters. Overall, 21.15% of samples displayed cytoplasmic staining of Bcl2 protein expression. However, 9.6% of patients had DAPK promoter hypermethylation and Bcl2 over expression with poor prognosis and metastasis. Our findings demonstrated that hypermethylation of the DAPK gene and Bcl2 expression leads to TCC recurrence and may have clinical implications for TCC prognosis and therapy.

Key words: Bcl2, Bladder Cancer Recurrence, DAPK, methylation, TCC.

INTRODUCTION

Bladder cancer is the fourth and eighth most common malignancy in the world and the third and ninth in Iran, respectively, for men and women (Akbari, 2008); the number of diagnosed cases is increasing annually (Cancer Facts & Figures available at www. cancer.org). There are two major subtypes of bladder cancer: non-muscle-invasive cancers, which frequently recur and often have a good prognosis, and muscle-invasive cancers which have a poor prognosis (Dinney *et al.*, 2004; Wu, 2005) and are mostly incurable (> 90%) when there is local or distal metastasis. Fifteen percent of non-muscle-invasive bladder cancers progress to the muscle-invasive disease (Chan *et al.*, 2009).

About 95% of bladder neoplasms are transitional cell carcinomas. The remainder are squamous tumors, adenocarcinomas, and other subtypes (Silverman *et*

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al., 1996). Both tumor grade and stage of urothelial carcinoma are highly correlated with recurrence, progression and patient survival rates (Soloway *et al.*, 2003). Tumor grade classification is determined by cytological features while tumor stage is related to invasiveness and metastasis (Schned *et al.*, 2008)*.* Bladder cancers are a mixture of heterogeneous cell populations and several factors dictate their recurrence, progression and patient survival (Kim *et al.*, 2007).

The exact etiology of bladder cancer is still unknown. However, several risk factors appear to be involved in its pathogenesis, including cigarette smoking (considered the main cause of bladder cancer) (Raveh *et al.*, 2001; Sasco *et al.*, 2004), genetic predisposition, occupational exposures to aromatic amines, polycyclic aromatic hydrocarbons, little water consumption, immunosuppression, cyclophosphomide, radiotherapy, arsenic and chronic cystitis (Esteller, 2002; Sasco *et al.*, 2004; Zarzour *et al.*, 2008).

The initiation and progression of cancer is associated with both genetic and epigenetic changes. Epigenetic changes, being independent of primary DNA sequence, play a key role in cancer initiation as well as tumor progression (Dong *et al.*, 2001; Raval *et al.*, 2007).

While the importance of epigenetic alterations in cancer is well recognized, the epigenetic changes are a more recent subject in this regard. The term 'epigenetics' is defined as all meiotically and mitotically heritable changes in gene expression that are not coded in the DNA sequence itself (Egger *et al.*, 2004). There are many contributing factors in the establishment and maintenance of epigenetic control, including promoter hypermethylation, methyl-binding proteins, histone deacetylases, DNA methyltransferases and chromatin state (Takai & Jones, 2002). Found in early stages of carcinogenesis, aberrant methylation of CpG islands is one of the features of human cancers (Egger *et al.*, 2004). Genes which are frequently inactivated by promoter region hypermethylation in cancer cells include MGMT (DNA repair), p16INK4a, p15INK4b (cell cycle), DAPK (apoptosis), and GSTP1 (detoxification) (Esteller, 2002; Groenendijk *et al.*, 2011).

The DAPK gene located on chromosome 9p34.1, encodes Ca^+/calmodulin-regulated serine/threonine kinase involved in apoptosis induction and suppression of tumor growth (D'Cruz *et al.*, 2001; Zhang *et al.*, 2007). DAPK expression is lost in many human cancers, often due to silencing by DNA hypermethylation (Reddy *et al.*, 2003; Wu, 2005). It acts as a posi-

tive regulator of apoptosis in part via phosphorylation of p53, as well as the RAS/RAF/MAPK pathway via phosphorylation after activation of ERK (RAS-extracellular signal kinase). Loss of DAPK1 function is common in different cancers such as ovarian cancer (Brown & Palmer, 2009), endometrial cancer (Girling *et al.*, 2010), and hematological cancers (Raval *et al.*, 2007). Furthermore, the loss of DAPK protein by promoter methylation is associated with advanced tumor invasion depth and advanced tumor stages (D'Cruz *et al.*, 2001; Kuester *et al.*, 2007; Wood *et al.*, 2007).

Bcl2 can be both oncogenic and tumor suppressive due to its anti-apoptotic activity and its function to suppress proliferation, respectively (Strasser *et al.*, 1990). This gene encodes an integral outer mitochondrial membrane protein that prevents the apoptotic death of some cells such as lymphocytes and regulates cell death by controlling the mitochondrial membrane permeability (Oltval *et al.*, 1993; Deng *et al.*, 2006).

Studies suggest that the suppression of the Bcl2 gene increases apoptosis (Motoyama *et al.*, 1995) and over-expression of the gene, even in the absence of trophic factors, sustains cell survival (Rohn *et al.*, 2008; Davies & Rubinsztein, 2011). Apoptosis is modulated by several proteins or antigens such as the Bcl2 family, DAPK and RASSF1. The interactions between effector and suppressor molecules play a key role in the regulation of apoptosis. Deregulation of the genes controlling apoptosis may contribute to tumorigenesis by reducing the cell death rate and facilitating the accumulation of other genetic defects (Delia *et al.*, 1992). Thus, Bcl2 is a type of proto-oncogene that promotes cancer development by suppressing cell death rather than by stimulating proliferation (Selvakumaran *et al.*, 1994).

In this study, we sought to determine whether there was a correlation between methylation status of DAPK and Bcl2 expression, which influenced the risk of bladder cancer and the recurrence rate.

MATERIALS AND METHODS

Study subjects

Eighty histologically confirmed incidents of Transitional of Cell Carcinoma (TCC) of the bladder specimens were obtained from the Department of Urology, Hasheminejad Hospital, Tehran University of Medical sciences. None of the patients had received chemotherapy or radiation before inclusion. The criteria for

the selection of patient samples were based on clinical proforma, pathological, and histo-pathological records. This study was approved by the Ethical Committee of the Institute. Bladder tumor samples were examined by a pathologist and classified according to the 1973 and 2004 WHO guidelines for bladder tumors (www.uroweb.org). Patients data were collected through interview where demographic features, clinical details, and smoking status were recorded using a standard clinical questionnaire. Control samples were obtained from healthy individuals urine samples who had come to the hospital for routine check up and who had no prior history of cancer, pre-cancerous lesions, or acute inflammatory disease. Cases and controls were matched by age, sex, and socio-economic status. Most of the subjects had completed their primary education. Smoking habits were also studied among the subjects. Subjects who had smoked at least for 5 years or more during their lifetimes were defined as smokers.

Methods

Samples of bladder tissues for each case were obtained from formalin-fixed paraffin-embedded blocks and from urine specimens for controls. The tissues were cut into 4-μm sections and hematoxylin-eosin (HE) stained for microdissection. For DNA extraction, the tissue fragments and urine specimens were processed using the QIAamp DNA extraction kit (Qiagen, Germany) according to the manufacturer's instructions. DNA samples were stored at –20°C until analysis.

Bisulphite treatment of DNA and Methylation Specific PCR (MS-PCR)

DAPK promoter methylation was analyzed in tumor and control samples. DNA methylation status in the CpG island of DAPK was determined by MS-PCR, performed as reported by Herman *et al.* (1996).

Bisulphite conversion was performed with the EpiTect Bisulfite Kit (Qiagen, Germany) on 1 μg normal or tumor DNA. MyoD amplification, with primers selected in an unmethylated region, served as the bisulphite treatment control. Primer sequences and PCR conditions for DAPK and MyoD, listed in Table 1, have been reported elsewhere (Esteller *et al.*, 1999; Kawakami *et al.*, 2000).

EpiTect positive control DNA (Qiagen, Germany) and re-suspended DNA were used in a PCR reaction. The reaction was carried out in a final volume of 50 μl containing 15 ng of bisulphite treated DNA, 1.5 mmol l^{-1} of MgCl$_2$, 0.2 mmol l^{-1} of each dNTP (Fermentas, USA), 200 nM of each primer, and 2.5 units of hot start Taq polymerase (SinaClon, Iran). DNA was amplified during 30 cycles with an initial denaturation of 5 min at 95°C and a final extension of 5 min at 72°C. The cycling program consisted of 30 sec denaturation at 95°C, 30 sec annealing at 60°C, and 30 sec extension at 72°C. PCR products were analyzed on a 2% agarose gel. DAPK methylated and unmethylated PCR products were 98 and 106 bp, respectively (Fig. 1). In order to avoid misinterpretation of methylated and unmethylated products, reaction mixtures for methylated and unmethylated primers were prepared separately.

Quantitative and qualitative assessment of immunohistochemistry

Fifty two out of 80 representative paraffin blocks were serially cut at 4 μm thick, de-paraffinized in xylen, dehydrated in graded ethanol, and washed for 5 min with phosphate-buffered saline. For antigen retrieval, the sections were immersed in 10 mM citrate buffer (pH 6.0) and boiled. Endogenous peroxidase was blocked by incubation of the slides for 30 min with 3% hydrogen peroxide in methanol. Sections were incubated with primary mouse monoclonal anti-Human Bcl2 Oncoprotein Clone 124 (Dako North America, Inc.) at room temperature. Immunohistochemical stains were performed using the high-sensitive polymer-based system (Dako EnVision+, Dual Link System Peroxidase) with Diaminobenzidine substrate solution as the chromogen. Sections were counterstained with Mayer's hematoxylin, dehydrated, and mounted following the standard procedure.

Two independent pathologists, blinded to clinical characteristics, evaluated all immunoassayed slides. The same area on each slide was examined. Each marker was scored in randomly selected fields of view by light microscopy (×400). The entire section was examined to find the foci with highest staining. The percentage of positive cells was determined from a mean of 500 cells per slide. Bcl2 positive cases were defined as those that showed more than 1% staining of tumor cells. Infiltrating lymphocytes and normal basal layer cells were used as internal positive controls for Bcl2 (Figs 2 and 3).

Statistical analysis

Hardy-Weinberg equilibrium was tested by a goodness-of-fit χ^2 test to compare the observed methyla-

TABLE 1. Primers for methylation-specific PCR

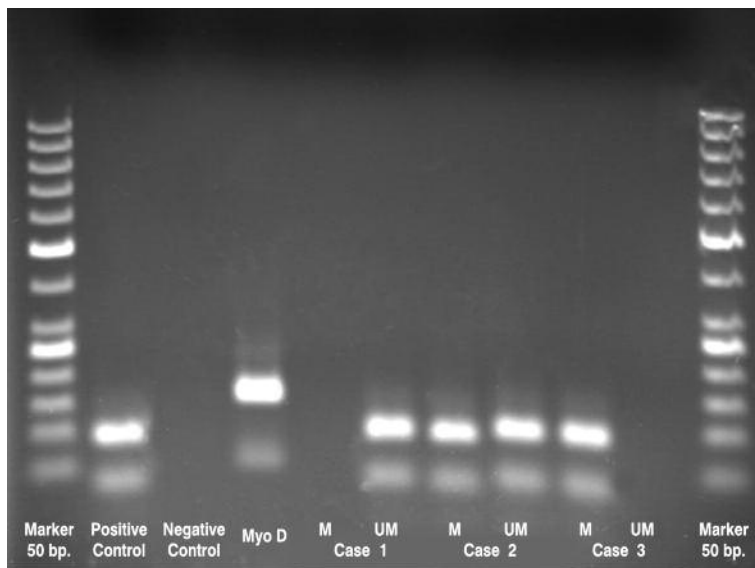| Gene	Primer (5′-3′)	Product size (bp)
DAPK M	GGATAGTCGGATCGAGTTAACGTC CCCTCCCAAACGCCGA	98
DAPK UM	GGAGGATAGTTGGATTGAGTTAATGTT CAAATCCCTCCCAAACACCAA	106
Myo D	TGATTAATTTAGATTGGGTTTAGAGAAGGA CCAACTCCAAATCCCCTCTCTAT	152

FIG. 1. Methylation analysis of DAPK promoter region in tumor tissues of bladder cancer patients by MS-PCR. Agarose gel electrophoresis of representative cases is shown. In Case 1: DAPK was unmethylated. In Case 2: DAPK was both methylated and unmethylated. In Case 3: DAPK was methylated. Myo D amplification served as the bisulfite treatment control. EpiTect methylated DNA (Qiagen, Germany) with DAPK promoter methylation served as the positive and normal human bladder epithelium (NBE) as the negative control. M and UM indicate methylation and unmethylation, respectively. Methylation-specific PCR products were run on a 2% agarose gel.
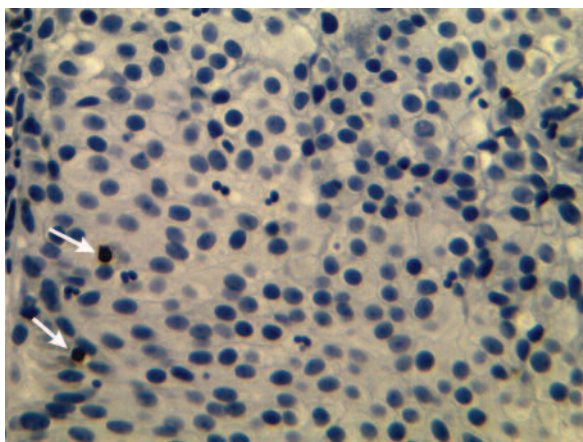

FIG. 2. Immunohistochemical detection of Bcl2 expression. Cytoplasmic positivity for Bcl2 (> 1%) in virtually all transitional cell carcinoma (×400). Arrows show epithelial cells expressing Bcl2.
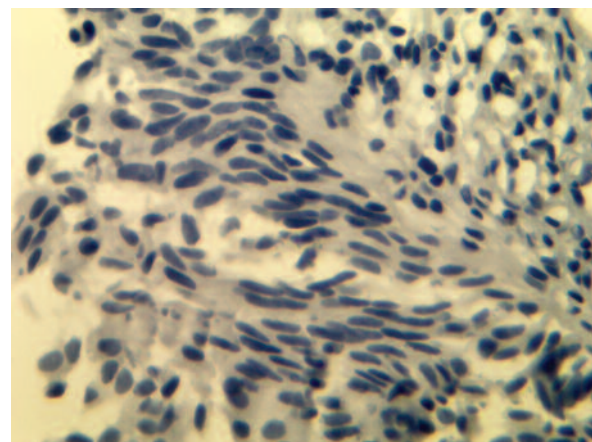

FIG. 3. Immunohistochemical detection of Bcl2 expression. Cytoplasmic positivity for Bcl2 (< 1%) in transitional cell carcinoma (×400).

tion status frequencies with the expected methylation status frequencies among the cases and controls. The power calculations were conducted at 80% with a significance level of 0.05. The data showed a normal distribution on applying one-sample Kolmogorov-Smirnov Z test when age was taken as the grouping variable. The data were age matched, as confirmed by t-test. The odds ratios (ORs) and 95% confidence intervals (CIs) were obtained using χ^2 test and Fisher's exact test for categorical variables. The ORs were calculated without adjustment for potential confounders, that is, sex and smoking. To achieve an adequate sample size with the power of study at 80%, the various tumor stages were merged into two groups, superficial (Ta + T1) and muscle-invasive (T2 + T3 + T4). Both additive and dominant modes of inheritance were considered. The *p*-values were two-sided (*p* < 0.05 were considered significant). All analyses were performed using SPSS v.15.0 (IBM, NY, USA) and Epi Info v.3.4.3 (WHO, GA, USA).

RESULTS

Demographic characteristics of cases and controls are summarized in Table 2. The hypermethylation of DAPK gene was studied in 80 patients [66 (82.5%) males and 14 (17.5%) females] with mean age of 58.19 years. The control group consisted of 70 (87.5%) males and 10 (12.5%) healthy females with mean age of 55.45 years. Current smokers were 62.5% of patients and 32.50% of controls.

No hypermethylation of DAPK was detected in DNA samples of 80 cancer-free individuals. DAPK

methylation was found in 29 out of 80 (36.25%) tissue samples of bladder cancer patients by MS-PCR (Fig. 1). In tissue samples of bladder cancer patients, the incidence of DAPK hypermethylation significantly increased (OR = 1.43, 95% CI = 1.12−1.83, *p* = 0.01) with an increase in age.

Clinicopathological analysis revealed that DAPK hypermethylation in tissue samples was not significantly associated with an increase in grade, but there was significant association in cases with tumor recurrence when compared to cases having no recurrences (OR = 3.52, 95% CI = 1.82−6.79, *p* = 0.0001). Smoking status showed significant association with DAPK promoter hypermethylation in tissue samples of the current smokers patients (OR = 1.5, 95% CI = 1.09−2.06, *p* = 0.03) when compared with non-smokers.

Overall, 78.85% of the patients showed very low or undetectable levels of Bcl2 expression and 21.15% displayed cytoplasmic staining of Bcl2 expression (Figs 2 and 3). To determine whether the Bcl2-positive and -negative patient groups were similar with respect to established prognostic characteristics, the two groups were compared. There were no significant associations between Bcl2 expression and age, grade, or metastases found. However, 9.6% of patients had DAPK promoter hypermethylation and Bcl2 over expression with poor prognosis and metastasis.

DISCUSSION

Epigenetic alterations produce changes in gene expression without having an effect on the DNA coding sequence itself. Promoter region hypermethylation is known to be an early event in carcinogenesis (Dammann *et al.*, 2000, 2005; Agathanggelou *et al.*, 2005; Cui *et al.*, 2007; Board *et al.*, 2007). Alteration in cell cycle, oncogenic and apoptotic proteins are key factors that determine the biological behavior of bladder cancer (Pich *et al.*, 2003).

The methylation of CpG islands located within the promoter element generally results in a decrease or loss in protein expression (Baylin *et al.*, 1998; Delgado *et al.*, 1998). De novo CpG island methylation has been reported in a wide spectrum of human cancers (e.g. colon, stomach, pancreas, liver kidney, bladder, brain, leukemia and lymphomas), however, the pattern of the methylation profile differs from one tumor type to another (Esteller *et al.*, 2001).

The results of this study indicate that aberrant promoter hypermethylation of the DAPK gene occurs frequently in bladder cancer tumors. Of 80 pa-

TABLE 2. Demographic characteristic of cases with bladder cancer and healthy controls

Variable	Cases	Controls
Age (year)		
Mean (± SD)	58.19 (±11.59)	55.45 (±10.13)
Range	28-86	30-75
Sex		
Male	66 (82.5%)	70 (87.5%)
Female	14 (17.5%)	10 (12.5%)
Smoking habit		
Current smokers	50 (62.5%)	26 (32.50%)
Non-smokers	30 (37.5%)	54 (67.50%)
Grade		
Low grade	69 (86.25%)	
High grade	11 (13.75%)	

tients with bladder cancer, we detected a strong association between promoter hypermethylation of DAPK (36.25%) and bladder cancer. Thus, this represents a probable marker for bladder cancer detection. In other studies it has been shown that DAPK expression is frequently lost in urinary bladder, breast, B-cell neoplasma, and renal cell carcinoma, due to promoter hypermethylation (Stephen *et al.*, 2009). Clinicopathological analysis has revealed that there is significant association between methylation status of DAPK1 and risk of tumor recurrence, an important prognosis factor for recurrence (Tada *et al.*, 2002). Loss of DAPK protein expression is associated with advanced tumor invasiveness and tumor stage (Costello *et al.*, 2000).

Several lines of evidence indicate that DAPK plays an important role in tumor suppression. The expression of DAPK is frequently lost in various human cancer cell lines and tumor tissues. This loss of expression leads to recurrence and/or metastasis of several human cancers (Raveh & Kimchi, 2001; Gozuacik & Kimchi, 2004). It is well documented that DAPK elicits proapoptotic activity. DAPK blocks random migration by suppressing directional resistance. During directed migration, DAPK acts as a strong blocker of cell polarization. Because these effects of DAPK are detected without any sign of apoptosis in cells, it is concluded that they are independent of proapoptotic activity (Kuo *et al.*, 2006). Also, the motility-inhibitory effect of DAPK is particularly important to suppress tumors that have escaped from DAPK-induced apoptosis. The migration/invasion inhibitory function of DAPK might be more significant in clinical results than its proapoptotic role (Raveh & Kimchi, 2001; Gozuacik & Kimchi, 2004).

In our study, 9.6% of bladder cancer patients have shown both hypermethylation of DAPK and Bcl2 over expression, all of whom had poor prognosis and developed metastases. Over-expression of Bcl2 has been reported in a wide variety of cancers including prostate, colorectal, lung, renal, bladder, and leukemia (Cho *et al.*, 2006). Furthermore, hypermethylation of DAPK leads to bladder cancer recurrence (Tada *et al.*, 2002).

Bcl2 was initially categorized as a proto-oncogene for its anti-apoptotic function (Reed, 1997). It is also involved in regulating DNA damage and repair, and may be associated with genomic instability and mutagenesis, both hallmarks of cancer (Distelhorst & Shore, 2004; Dumay *et al.*, 2006; Placzek *et al.*, 2010). Bcl2 expression in endothelial cells appeared to enhance tumor metastasis (Kumar *et al.*, 2008). Several studies have provided conclusive evidence that elevation in Bcl2 expression cause resistance to chemotherapy and radiotherapy and increase cell proliferation (Reed, 1999). In another study, the positive immunostaining of Bcl2 was observed in 69% of bladder cancers and 75% of patients had suffered from high-grade tumors (Atuğ *et al.*, 1998).

There was significant association of DAPK promoter hypermethylation in tissue samples from smokers compared to non-smokers. Cigarette or tobacco smoke accounts for about 65% of bladder cancer risk in men and 20-30% in women (Alguacil *et al.*, 2011). The metabolism of tobacco carcinogens generates free radicals which may cause further oxidative damage (Samanic *et al.*, 2006). Other findings suggest that, for an equal total exposure (in pack-years), smoking at lower intensity and longer duration is more harmful than smoking at higher intensity and shorter duration (Lubin *et al.*, 2007; Stern *et al.*, 2009).

Age is also an important factor of hypermethylation status in normal tissue (Lubin *et al.*, 2008), such that methylation has been suggested as a molecular "clock" to predict the age of the tissue (Hartge *et al.*, 1987). In our study we found that there was significant association between hypermethylation status of DAPK and an increase in age ($p = 0.01$).

It is now generally accepted that macromolecular damage due to mitochondrially-produced reactive oxygen species is the key cause of aging and eventual death of multicellular organisms. These molecules mostly affect old postmitotic cells including neurons and cardiac myocytes. These cells are rarely, or not at all, replaced during life and can be as old as the whole organism (Ahuja *et al.*, 1998). For the genes which are decreasing during life, this is not the case. Genes down regulated with aging in multiple tissues were more commonly associated with metabolic processes, chromosome organization/biogenesis, cell division and cycle, mRNA processing and splicing, and protein folding (Kim *et al.*, 2005).

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