Epigenetic Markers for Bladder Cancer in Urine

Wun-Jae Kim[1] and Yong-June Kim[2]

[1]Department of Urology, Chungbuk National University, College of Medicine and Institute for Tumor Research, 62 Kaeshin-dong, Heungduk-gu, Cheongju 361-763 South Korea.
[2]Department of Urology, Chungbuk National University College of Medicine, Cheongju, South Korea.

Abstract: Many tumor markers for bladder cancer have been evaluated for use in detecting and monitoring bladder cancers tissue specimens, bladder washes, and urine specimens. However, none of the biomarkers reported to date has shown sufficient sensitivity and specificity to detect the entire spectrum of bladder cancers in routine clinical practice. The limited value of the established prognostic markers demands analysis of new molecular parameters having the potential to predict the prognosis of bladder cancer patients, particularly, the high-risk patients at risk of cancer progression and recurrence. Abnormal methylation of CpG islands can efficiently repress transcription of the associated gene in a manner akin to mutations and deletions. Several tumor suppressor genes correlated with bladder cancer contain CpG islands in their promoters.

Markers for aberrant methylation may be a potential gateway for monitoring bladder cancer. Hypermethylation of several gene promoters was detected in urine sediment DNA from bladder cancer patients. Detection of DNA methylation in voided urine is feasible and noninvasive. Methylation is an important molecular mechanism in the development of bladder cancer and could be used as a prognostic and diagnostic marker. Aberrant patterns of epigenetic modification could, in the near future, be crucial indicators in cancer diagnosis, prognosis, and may additionally be good targets for developing novel therapies while maintaining quality of life.

Keywords: DNA methylation, Bladder cancer, Urine

Introduction

More than 90% of bladder cancers are transitional cell carcinomas and roughly 60% of bladder cancers are low-grade, superficial transitional cell carcinomas. After endoscopic resection, the majority of patients with these cancers develop cancer recurrences, 16% to 25% with high-grade cancers. Approximately 10% of patients with superficial bladder cancers subsequently develop invasive or metastatic cancers. Almost 25% of patients with newly diagnosed bladder cancer have muscle-invasive cancers, the vast majority being histologically high grade cancers. Almost 50% of patients with muscle-invasive bladder cancer already have occult distant metastases (Messing, 2002). Therefore, the frequent recurrence after transurethral resection of superficial bladder cancer and subsequent cancer progression are problems for both patients and urologists.

Cystoscopic examination is the gold standard to diagnose bladder cancer, but is costly, incurs substantial patient discomfort, and has variable sensitivity. The development of highly reliable, noninvasive tools for bladder cancer diagnosis would facilitate early detection and help to define the role of molecular markers in prognostic evaluation at the time of initial diagnosis of patients with bladder cancer.

All of the urothelial cells, proteins, and metabolites in the urinary tract can be isolated noninvasively from the urine and analyzed to detect disease in the urinary tract. Cytological analysis of voided urine has been the standard noninvasive method for cancer detection. However, the sensitivity is low, especially for low grade transitional cell carcinomas. More sensitive, non-invasive methods are required and many urine-based tumor markers have been developed for use in detecting and monitoring bladder cancers (Sozen et al. 1999; Priolo et al. 2001; Mahnert et al. 1999; Heicappell et al. 2000; Ramakumar et al. 1999; Johnston and Morales, 1999; Bubendorf et al. 2001; Friedrich et al. 2003; Feil et al. 2003; Lokeshwar et al. 2005; Mian et al. 1999; Giannopoulos et al. 2001). The Food and Drug Administration (FDA) has already accepted some of these tumor marker tests for use in routine patient care. Initial studies with new markers are usually promising, but subsequent reports often fail to show comparable results (van Rhijn et al. 2005).

Correspondence: Wun-Jae Kim, M.D., Ph.D., Department of Urology, Chungbuk National University, College of Medicine and Institute for Tumor Research, 62 Kaeshin-dong, Heungduk-gu, Cheongju 361-763, South Korea. Tel: 82-043-269-6371; FAX: 82-043-271-7716; Email: wjkim@chungbuk.ac.kr

The challenge for the urologist is to develop rational surveillance protocols that provide cost-effective, noninvasive monitoring for low-risk patients, while using a more active approach to identify high-risk refractory cancers before they metastasize. Methylation patterns are established during development and, normally, are maintained throughout the life of an individual. Consequently, DNA methylation is a key regulator of gene transcription and genomic stability, and alteration of DNA methylation is one of the most consistent epigenetic changes in human cancers. Hypermethylation of promoter regions of tumor suppressor genes is now the most well categorized epigenetic change in human neoplasias (Laird, 2003). In many cases, aberrant methylation of CpG island within genes has been correlated with a loss of gene expression, and it is proposed that DNA methylation provides an alternate pathway to gene deletion or mutation for the loss of tumor suppressor gene function. Markers for aberrant methylation may represent a promising avenue for monitoring the onset and progression of cancer. Aberrant promoter methylation has been described for several genes in various malignant diseases, and each tumor type may have its own distinct pattern of methylation (Costello et al. 2000; Esteller et al. 2001). In transitional cell carcinoma of the bladder, hypermethylation of CpG islands near the promoter region and decreased expression of tumor suppressor genes such as *RUNX3*, *p16,* and *E-cadherin* have been reported (Kim et al. 2005; Maruyama et al. 2001; Muto et al. 2000). Several studies have demonstrated that hypermethylation of various gene promoters was detectable in DNA isolated from bodily fluids, including urine sediment DNA from bladder cancer patients (Chan et al. 2002; Valenzuela et al. 2002). This article focuses on the prognostic relevance of DNA promoter hypermethylation detected in urine obtained from bladder cancer patients.

Conventional Biomarkers in Urine

In bladder cancer patients, lifelong surveillance is required to detect subsequent tumor recurrences. Many potential tumor markers for bladder cancer have been evaluated for detecting and monitoring the disease in serum, bladder washes, and urine specimens. Development of accurate and noninvasive bladder tumor markers is essential for screening, initial diagnosis, monitoring for recurrence, detection of early progression, and prediction of prognosis, without increasing the frequency of invasive and costly diagnostic procedures. Current patient monitoring protocols generally consist of cystoscopic evaluations and urine cytology every 3–4 months for the first two years and at longer intervals in subsequent years. Cytological examination of voided urine is a highly specific, noninvasive adjunct to cystoscopy. It has good sensitivity for detection of high-grade bladder cancers, but poor sensitivity for low-grade cancers. Furthermore, the accuracy of cytology is dependent upon the level of expertise of the pathologist (Sherman et al. 1984). Thus, noninvasive, objective, and accurate biomarkers are needed not only for the primary detection of bladder cancer, but also for monitoring the disease. The recent emergence of sensitive markers for bladder cancer has provided new opportunities for early bladder cancer detection. There are currently more than 20 urinary markers from various stages of disease progression. The FDA has already approved several urine tests for monitoring patients with bladder cancer, including the bladder tumor antigen (BTA) *stat* test, the BTA TRAK test, the fibrinogen–fibrin degradation products (FDP) test, UroVysion, ImmunoCyt, and the nuclear matrix protein-22 (NMP22) assay (Table 1). In general, each of these markers has better sensitivity but lower specificity than cytology, and must still be used as an adjunct to cystoscopy. Discrepancies among laboratories in sample handling, cutoffs, and the issue of specificity in nonmalignant urological diseases still pose a dilemma for application of these assays as routine tests in the clinical setting (Lotan and Roehrborn, 2003). None of the biomarkers reported to date has shown sufficient sensitivity and specificity in detecting the spectrum of bladder cancer diseases assessed in routine clinical practice (van Rhijn et al. 2005). The limited value of the established prognostic markers requires analysis of new molecular indicators having the potential to predict the prognosis of bladder cancer patients, particularly, high-risk patients at risk of cancer progression and recurrence.

Methylation Markers in Urine

Tumorigenesis is a multistep process that results from the accumulation and interplay of genetic mutations and epigenetic changes. The inheritance of information on the basis of gene expression levels is known as epigenetics, as opposed to

Table 1. Currently available urinary markers for bladder cancer.

Test	Marker	Sensitivity (%)	Specificity (%)	References
BTA *Stat*	Human complement factor H related protein	60–70	50–75	(Sozen et al. 1999; Priolo et al. 2001; Mahnert et al.1999; Heicappell et al. 2000)
BTA TRAK	Human complement factor H related protein	60–70	50–75	(Sozen et al. 1999; Priolo et al. 2001; Mahnert et al. 1999; Heicappell et al. 2000)
FDPs	FDPs	78–91	75–90	(Ramakumar et al. 1999; Johnston and Morales, 1999)
UroVysion	Chromosomal probes	70–100	90	(Bubendorf et al. 2001; Friedrich et al. 2003)
ImmunoCyt	High molecular weight CEA and mucins	70–95	70–85	(Feil et al 2003; Lokeshwar et al. 2005; Mian et al. 1999)
NMP22	Nuclear mitotic apparatus Protein	60–75	70–85	(Sozen et al. 1999; Lee, 2001; Giannopoulos et al. 2001; Ramakumar et al. 1999).

genetics, which refers to the information inherited on the basis of the gene sequence. DNA methylation is an epigenetic mechanism used for long-term silencing of gene expression. It can maintain differential gene expression patterns in a tissue-specific and developmental-stage-specific manner. The direct relationship between the density of methylated cytosine residues in CpG islands and local transcriptional inactivation has been widely documented (Jones and Laird, 1999). Transcriptional repression by DNA methylation is mediated by changes in chromatin structure. Specific proteins bound to methylated DNA recruit a complex containing transcriptional corepressors and histone deacetylases (Baylin et al. 2001). Histone deacetylation results in chromatin compaction and, hence, transcriptional inhibition.

Inactivation of gene expression by abnormal methylation of CpG islands can act as a ''hit'' for cancer generation (Jones and Laird, 1999; Baylin and Herman, 2000). Thus, alteration of DNA methylation in CpG islands is emerging as a key event in the inheritance of transcriptionally repressed regions of the genome. Many tumor suppressor genes contain CpG islands and show evidence of methylation specific silencing. Several genes, including *p16*, *RARß*, *E-cadherin*, *AUTHOR—E-cadherin, correct? DAPK*, and *RASSF1A*, have been reported to undergo methylation in bladder cancer (Tada et al. 2002; Chan et al. 2002; Dulaimi et al. 2004; Lee et al. 2001; Maruyama et al. 2001; Chan et al. 2003; Orlow et al. 1999). In some of these tumors, hypermethylation is associated with loss of heterozygosity; in others, hypermethylation affects both alleles.

Aberrant hypermethylation events can occur early in tumorigenesis, predisposing cells to malignant transformation. Moreover, promoter hypermethylation of CpG islands is strongly associated with tumor development, stage, recurrence, progression, and survival in transitional cell carcinomas of the urinary bladder (Kim et al. 2005; Catto et al. 2005). We have demonstrated that *RUNX3* methylation confers a 100-fold increase in the risk for bladder cancer development (OR, 107.55). *RUNX3* methylation was also associated with cancer stage (OR, 2.95), recurrence (OR, 3.70), and progression (OR, 5.63), suggesting that RUNX3 is required not only to inhibit cancer initiation but also to suppress aggressiveness in primary bladder cancers (Kim et al. 2005). Although various diagnostic markers for bladder cancer development, recurrence, and progression have been reported, none are adequate to predict the behavior of most tumors. The methylation status of *RUNX3* could be a better diagnostic marker for bladder cancer than previously described markers.

Catto et al. (2005) have analyzed hypermethylation at 11 CpG islands in a large cohort of upper urinary tract transitional cell carcinomas (UTTs) and lower tract (bladder) urothelial carcinomas (UCs), and have provided some interesting insights into the differential epigenetic features of the two types of malignancies. Despite morphological similarities between these cancers, more extensive promoter hypermethylation was found in UTTs (96%) than UCs (76%). Compared to tumors

without methylation, the presence of methylation was significantly associated with advanced stage, high tumor progression rates, and increased mortality rates. These findings strongly suggest that patterns of promoter hypermethylation are causally associated with bladder cancer and that methylation status could be a useful diagnostic and prognostic marker for bladder cancer in the clinical setting, as well as a therapeutic target for treatment of bladder cancers.

Because some genetic and epigenetic events will occur early in the disease process, molecular diagnosis may allow detection before symptomatic or overt radiographic manifestations. Thus, from a clinical point of view the most promising application for methylation analysis is the early detection of cancers or the utilization of methylation as a prognostic marker. Screening of bodily fluids such as urine may ultimately provide a truly noninvasive diagnostic modality, thereby limiting the need for current imaging techniques that provide anatomical details without definitive pathological correlations. Bodily fluids surrounding or drained from organs from patients with various solid malignancies have been successfully used in methylation specific PCR based detection. These epigenetic changes have been detected in serum (Sanchez-Cespedes et al. 2000), sputum (Belinsky et al. 1998), and urine (Goessl et al. 2000). Hypermethylation of several gene promoters correlated with bladder cancer in DNA isolated from cancer tissues and from urine sediment has been reported (Table 2). These studies revealed that detection of aberrant promoter methylation in urine is feasible and appears to be more sensitive than conventional cytology.

Chan et al. (2002) investigated the methylation state of 7 genes (*RARß, DAPK, E-cadherin, p16, p15, GSTP1,* and *MGMT)* in 22 voided urine samples from bladder cancer patients and 17 control samples. The frequency of methylation in the bladder cancer samples was 45.5% for *DAPK*, 68.2% for *RARß*, 59.1% for *E-cadherin*, and 13.6% for *p16*. Methylation of any one of these four genes could be detected in 90.9% of the urine samples, whereas urine cytology was able to detect cancer cells in only 45.5% of the samples. This difference was more striking in low-grade cases (100% versus 11.1%), where conventional urine cytology is known to have a low sensitivity. Methylation could only be detected in those patients whose tumor tissue also showed gene methylation and no false positives were detected. No methylated copies of *E-cadherin, DAPK,* or *p16* were detected in normal urine (100% specificity). Detecting combinations of methylation markers, however, had a lower specificity, which was related to the presence of methylated *RARß* in the normal urine controls (23.5%).

Dulaimi et al. (2004) examined the methylation status of tumor suppressor genes (*APC, RASSF1A*, $p14^{ARF}$) in matched samples of sediment DNA from urine specimens obtained before and after surgery from 45 bladder cancer patients, and in normal and benign control DNA samples. Hypermethylation of at least one of the three tumor suppressor genes was found in the matched urine DNA from 39 of 45 patients (87% sensitivity; 100% specificity), including 16 cases that had negative cytology. Hypermethylation (91%) was found more commonly than positive cytology (50%) in urine samples.

Friedrich et al. (2004) investigated DNA methylation of apoptosis-associated genes (*DAPK*, *BCL2*, *TERT*, *EDNRB*, *RASSF1A*, and *TNFRSF25*) in urine sediments. The combined methylation analysis of three genes (*DAPK*, *BCL2*, and *TERT*) provided a high sensitivity (78%) and specificity (100%) for detection of bladder cancer. However, methylation markers such as *EDNRB*, *RASSF1A*, and *TNFRSF25* may not be useful in detection of bladder cancer, since these regions were also methylated in cancer-free individuals.

The feasibility of detecting DNA hypermethylation in voided urine and its potential role as a tumor marker for bladder cancer has been recently reported (Hoque et al. 2006). In this study, a quantitative real-time PCR assay was introduced to examine urine sediment DNA from 175 patients with bladder cancer and 94 age-matched control subjects for promoter hypermethylation of nine genes (*APC*, $p14^{ARF}$, *CDH1*, *GSTP1*, *MGMT*, *CDKN2A*, *RARβ2*, *RASSF1A*, and *TIMP3*). Compared to conventional methylation-specific PCR, the quantitative analysis of PCR products was critical for reproducible interpretation of the results, and the quantitative methylation-specific PCR assay used in this study provided a highly sensitive automated approach for the detection of methylated alleles. The combined methylation analysis of four genes (*CDKN2A*, $p14^{ARF}$, *MGMT*, and *GSTP1*) displayed 69% sensitivity and 100% specificity. For patients without aberrant methylation of any of these four genes, addition of a logistic regression score based on the remaining five genes

Table 2. Potential epigenetic markers in bladder cancer.

Marker	Chromosomal locus	References
APC	5q21-q22	(Dulaimi et al. 2004)
BCL2	18q21.3	(Friedrich et al. 2004)
CDH1 (*E-cadherin*)	16q22.1	(Maruyama et al. 2001; Chan et al. 2002)
CDKN2A	9p21	(Hoque et al. 2006)
DAPK	9q34.1	(Chan et al. 2002; Catto et al. 2005; Friedrich et al. 2004)
FHIT	3p14.2	(Maruyama et al. 2001)
GSTP1	11q13	(Hoque et al. 2006)
LNMA3	18q11.2	(Sathyanarayana et al. 2004)
LNMB3	1q32	(Sathyanarayana et al. 2004)
LNMC2	1q25-q31	(Sathyanarayana et al. 2004)
MGMT	10q26	(Hoque et al. 2006)
$p14^{ARF}$	9p21	(Dulaimi et al. 2004; Hoque et al 2006)
$p16^{INK4A}$	9p21	(Chan et al. 2002)
RASSF1A	3p21	(Catto et al. 2005; Dulaimi et al. 2004; Chan et al. 2003; Maruyama et al. 2001; Marsit et al. 2006)
RUNX3	1p36	(Kim et al. 2005)
TERT	5p15.33	(Friedrich et al. 2004)
TIM3	5q33.2	(Friedrich et al. 2005)

improved sensitivity from 69% to 82% but decreased the specificity from 100% to 96%. With regard to the association between clinicopathological parameters and the methylation patterns identified in the urine sediment DNA, promoter methylation of both $p14^{ARF}$ and *MGMT* was significantly associated with increasing tumor stage. Promoter methylation of $p14^{ARF}$, *MGMT*, *GSTP1* and *TIMP3* was significantly associated with invasive tumors. Promoter methylation of *GSTP1* and *RASSF1A* was significantly associated with positive cytology. Aberrant methylation of the nine genes examined in the urine sediment DNA of bladder cancer patients was not associated with any other clinical or demographic characteristics, including age at the time of diagnosis, sex, histological subtype, or tumor recurrence. The combined methylation marker approach provides evidence that increasing the number of markers in the assay panel increases the sensitivity, but also decreased the specificity of the assay while increasing the cost. These results imply that only an extension of the selected panel of methylation markers might result in higher sensitivity and specificity in methylation analysis of the urine. These studies suggest, therefore, that the combined methylation marker assay is a promising noninvasive diagnostic and monitoring tool for detection of noninvasive bladder cancers.

There are, however, a number of criticisms of the clinical and prognostic relevance of assays detecting promoter hypermethylation in the urine. First, the exact molecular mechanisms of DNA methylation in health and cancer remain to be elucidated. One uncertainty is the extent of aberrant DNA methylation in nonmalignant tissue and its increase with aging. In contrast to early reports indicating a lack of aberrant hypermethylation in normal tissues, promoter hypermethylation is often found in histologically normal tissues, and is correlated with aging (Issa, 2000). Aging-related methylation has been demonstrated for the *ER* gene by Issa et al. (1994) and for several other genes by Ahuja et al. (1998), which perhaps partly explains the direct correlation between aging and increased incidence of cancer. Susceptibility to aging-related aberrant hypermethylation differs among genes and tissues (Ahuja et al. 1998). Although little is known about the causes of aging-related aberrant hypermethylation, the phenomenon might be related to the accessibility of the tissue to exogenous chemicals (Issa, 2002). In addition, endogenously-produced chemicals, including reactive oxygen species, are suspected

as possible causes (Shen et al. 2002). Thus, tumor suppressor gene methylation can be found in exfoliated urinary cells from patients without cancer and increases in frequency with aging (Friedrich et al. 2004, Yates et al. 2006). In the near future, it may be possible that technology will be developed to discriminate between methylation patterns related to aging versus cancer. Secondly, several studies of promoter methylation produced different results. Eventually, new methodologies might explain these discrepancies, but these dissimilarities emphasize the need for standardized methodological protocols if molecular diagnostic tools are to be a useful component of routine clinical practice.

Third, some markers, such as *GSTP1*, can be methylated in both bladder and prostate cancer. *GSTP1* promoter methylation is an attractive prostate cancer biomarker because it is seldom observed in non-cancerous prostate tissue (Brooks et al. 1998; Henrique and Jeronimo, 2004). Furthermore, *GSTP1* hypermethylation is more frequent than bladder cancer (Chan et al. 2002; Maruyama et al. 2001). Even though a specific methylation marker in urine might be promising, the most powerful established detection tools, such as cystoscopy in bladder cancer and PSA in prostate cancer, should be first considered and methylation markers must still be used as adjuncts.

Additionally, discrepancies between methylation profiles in urine and surgical samples have been reported. In several published studies, paired urine and surgical specimens had a very high level of concordance (Chan et al. 2002; Chan et al. 2003). However, Pu et al. (2006) demonstrated that urine methylation profiles were quite different from those of the corresponding follow-up biopsy specimens. The most likely explanation for this discrepancy lies in the different sensitivities of the assays used. Urine is obviously a more heterogeneous sample than a bladder biopsy, since it may contain cells from different neoplastic clones throughout the bladder. Utilization of multiplex methylation specific PCR (MSP) or quantitative PCR assays on urine may lead to detection of rare cells from clones remote from the biopsy site. Conversely, methylation events detected in biopsy samples but not in urine may represent rare events within the biopsy sample, possibly in the deep layers of the urothelium, that are not represented in urine. Future studies should be focused on optimizing the sensitivity of MSP assays for the detection of clinically relevant urothelial lesions from urine samples. However, detecting DNA hypermethylation in voided urine remains promising in terms of early detection and surveillance of bladder cancer**.**

Conclusions

Transitional cell carcinomas of the urinary bladder have diverse biological and functional characteristics. Although current pathological and clinical variables provide important prognostic information, these variables are still limited in assessing the true malignant potential of most bladder cancers. A better understanding of the molecular mechanisms involved in carcinogenesis and cancer progression has provided a large number of molecular markers of bladder cancer having potential diagnostic and prognostic value. Cystoscopy is the mainstay for diagnosing bladder cancer, but is associated with high cost and patient discomfort. Cytology and many urine-based tumor markers provide minimal information for detecting and predicting the prognosis of bladder cancers. In contrast, promoter hypermethylation of CpG islands is strongly associated with tumor development, stage, recurrence, progression, and survival in transitional cell carcinomas of the urinary bladder. Detection of DNA methylation in voided urine is feasible and is also more sensitive than conventional urine cytology. Ultimately, all types of urological cancers may be screened in urine with a larger panel of hypermethylated genes. The panel could be easily extended in the future to simultaneously provide early detection and prognostic stratification, as well as providing novel targets for therapy. The epigenetic silencing of tumor suppressor genes is interesting from a clinical standpoint because it is possible to reverse epigenetic changes and restore gene function to a cell. In terms of treatment and prevention of bladder cancer, methylation markers might be more useful than conventional molecular markers. Treatment with DNA methylation inhibitors can restore the activities of dormant genes such as *RUNX3* and decrease the growth rate of cancer cells in a heritable fashion. It should therefore be possible to partially reverse the cancer phenotype by the use of methylation inhibitors. This will eventually lead to personalized target therapy tailored toward specific

molecular defects, thereby significantly lowering the morbidity associated with bladder cancer.

Acknowledgements

The present study was supported by Regional Industry Technology Department Project, Ministry of Commerce, Industry and Energy (Grant No. 10018327), and Ministry of Health and Welfare, Korea.

References

Ahuja, N., Li, Q., Mohan, A.L., Baylin, S.B. and Issa, J.P. 1998. Aging and DNA methylation in colorectal mucosa and cancer. *Cancer Res.*, 58:5489–94.

Baylin, S.B., Esteller, M. and Rountree, M.R. et al. 2001. Aberrant patterns of DNA methylation, chromatin formation and gene expression in cancer. *Hum. Mol. Genet.*, 10:687–92.

Baylin, S.B. and Herman, J.G. 2000. DNA hypermethylation in tumorigenesis: epigenetics joins genetics. *Trends Genet.*, 16:168–74.

Belinsky, S.A., Nikula, K.J., Palmisano, W.A. et al. 1998. Aberrant methylation of p16(INK4a) is an early event in lung cancer and a potential biomarker for early diagnosis. *Proc. Natl. Acad. Sci. U.S.A.*, 95:11891–6.

Brooks, J.D., Weinstein, M. and Lin, X. et al. 1998. CG island methylation changes near the GSTP1 gene in prostatic intraepithelial neoplasia. *Cancer Epidemiol. Biomarkers Prev.*, 7:531–6.

Bubendorf, L., Grilli, B. and Sauter, G. et al. 2001. Multiprobe FISH for enhanced detection of bladder cancer in voided urine specimens and bladder washings. *Am. J. Clin. Pathol.*, 116:79–86.

Catto, J.W., Azzouzi, A.R. and Rehman, I. et al. 2005. Promoter hypermethylation is associated with tumor location, stage, and subsequent progression in transitional cell carcinoma. *J. Clin. Oncol.*, 23:2903–10.

Chan, M.W., Chan, L.W. and Tang, N.L. et al. 2003. Frequent hypermethylation of promoter region of RASSF1A in tumor tissues and voided urine of urinary bladder cancer patients. *Int. J. Cancer*, 104:611–6.

Chan, M.W., Chan, L.W. and Tang, N.L. et al. 2002. Hypermethylation of multiple genes in tumor tissues and voided urine in urinary bladder cancer patients. *Clin. Cancer Res.*, 8:464–70.

Costello, J.F., Fruhwald, M.C. and Smiraglia, D.J. et al. 2000. Aberrant CpG-island methylation has non-random and tumour-type-specific patterns. *Nat. Genet.*, 24:132–8.

Dulaimi, E., Uzzo, R.G., Greenberg, R.E. and Al-Saleem, T., Cairns, P. 2004. Detection of bladder cancer in urine by a tumor suppressor gene hypermethylation panel. *Clin. Cancer Res.*, 10:1887–93.

Esteller, M., Corn, P.G., Baylin, S.B., Herman, J.G. 2001. A gene hypermethylation profile of human cancer. *Cancer Res.*, 61:3225–9.

Feil, G., Zumbragel, A. and Paulgen-Nelde, H.J. et al. 2003. Accuracy of the ImmunoCyt assay in the diagnosis of transitional cell carcinoma of the urinary bladder. *Anticancer Res.*, 23:963–7.

Friedrich, M.G., Chandrasoma, S. and Siegmund, K.D. et al. 2005. Prognostic relevance of methylation markers in patients with non-muscle invasive bladder carcinoma. *Eur. J. Cancer*, 41:2769–78.

Friedrich, M.G., Toma, M.I., Hellstern, A. et al. 2003. Comparison of multitarget fluorescence in situ hybridization in urine with other noninvasive tests for detecting bladder cancer. *BJU. Int.*, 92:911–4.

Friedrich, M.G., Weisenberger, D.J. and Cheng, J.C. et al. 2004. Detection of methylated apoptosis-associated genes in urine sediments of bladder cancer patients. *Clin. Cancer Res.*, 10:7457–65.

Giannopoulos, A., Manousakas, T. and Gounari, A. et al. 2001. Comparative evaluation of the diagnostic performance of the BTA stat test, NMP22 and urinary bladder cancer antigen for primary and recurrent bladder tumors. *J. Urol.*, 166:470–5.

Goessl, C., Krause, H. and Muller, M. et al. 2000. Fluorescent methylation-specific polymerase chain reaction for DNA-based detection of prostate cancer in bodily fluids. *Cancer Res*, 60:5941–5.

Heicappell, R., Muller, M., Fimmers, R. and Miller, K. 2000. Qualitative determination of urinary human complement factor H-related protein (hcfHrp) in patients with bladder cancer, healthy controls, and patients with benign urologic disease. *Urol. Int.*, 65:181–4.

Henrique, R., Jeronimo, C. 2004. Molecular detection of prostate cancer: a role for GSTP1 hypermethylation. *Eur. Urol.*, 46:660–9.

Hoque, M.O., Begum, S. and Topaloglu, O. et al. 2006. Quantitation of promoter methylation of multiple genes in urine DNA and bladder cancer detection. *J. Natl. Cancer Inst.*, 98:996–1004.

Issa, J.P. 2000. The epigenetics of colorectal cancer. *Ann. N.Y. Acad Sci.*, 910:140–53.

Issa, J.P. 2002. Epigenetic variation and human disease. *J. Nutr.*, 132 (Suppl 8):2388–92.

Issa, J.P., Ottaviano, Y.L. and Celano, P. et al. 1994. Methylation of the oestrogen receptor CpG island links ageing and neoplasia in human colon. *Nat. Genet.*, 7:536–40.

Johnston, B. and Morales, A. 1999. Re: Comparison of screening methods in the detection of bladder cancer. *J. Urol.*, 162:1388–9.

Jones, P.A. and Laird, P.W. 1999. Cancer epigenetics comes of age. *Nat. Genet.*, 21:163–7.

Kim, W.J., Kim, E.J. and Jeong, P. et al. 2005. RUNX3 inactivation by point mutations and aberrant DNA methylation in bladder tumors. *Cancer Res.*, 65:9347–54.

Laird, P.W. 2003. The power and the promise of DNA methylation markers. *Nat. Rev. Cancer*, 3:253–66.

Lee, K.H. 2001. Evaluation of the NMP22 test and comparison with voided urine cytology in the detection of bladder cancer. *Yonsei Med. J.*, 42:14–8.

Lee, M.G., Kim, H.Y. and Byun, D.S. et al. 2001. Frequent epigenetic inactivation of RASSF1A in human bladder carcinoma. *Cancer Res*, 61:6688–92.

Lokeshwar, V.B., Habuchi, T. and Grossman, H.B. et al. 2005. Bladder tumor markers beyond cytology: International Consensus Panel on bladder tumor markers. *Urology*, 66:35–63.

Lotan, Y. and Roehrborn, C.G. 2003. Sensitivity and specificity of commonly available bladder tumor markers versus cytology: results of a comprehensive literature review and meta-analyses. *Urology*, 61:109–18.

Mahnert, B., Tauber, S., Kriegmair, M. et al. 1999. BTA-TRAK—a useful diagnostic tool in urinary bladder cancer? *Anticancer Res.*, 19:2615–9.

Marsit, C.J., Karagas, M.R. and Danaee, H. et al. 2006. Carcinogen exposure and gene promoter hypermethylation in bladder cancer. *Carcinogenesis*, 27:112–6.

Maruyama, R., Toyooka, S. and Toyooka, K.O. et al. 2001. Aberrant promoter methylation profile of bladder cancer and its relationship to clinicopathological features. *Cancer Res.*, 61:8659–63.

Messing, E.M. 2002. Urothelial tumors of the urinary tract. In: Walsh, P.C., Retik, A.B., Vaughan, E.D., Wein, A.J. ed. Campbell's urology 8th ed. Pennsylvania: Saunders. p 2750–2751.

Mian, C., Pycha, A. and Wiener, H. et al. 1999. Immunocyt: a new tool for detecting transitional cell cancer of the urinary tract. *J. Urol.*, 161:1486–9.

Muto, S., Horie, S., Takahashi, S., Tomita, K., Kitamura, T. 2000. Genetic and epigenetic alterations in normal bladder epithelium in patients with metachronous bladder cancer. *Cancer Res.*, 60:4021–5.

Orlow, I., LaRue, H., Osman, I. et al. 1999. Deletions of the INK4A gene in superficial bladder tumors. Association with recurrence. *Am. J. Pathol.*, 155:105–13.

Pu, R.T., Laitala, L.E. and Clark, D.P. 2006. Methylation profiling of urothelial carcinoma in bladder biopsy and urine. *Acta. Cytol.*, 50:499–506.

Priolo, G., Gontero, P. and Martinasso, G. et al. 2001. Bladder tumor antigen assay as compared to voided urine cytology in the diagnosis of bladder cancer. *Clin. Chim. Acta.*, 305:47–53.

Ramakumar, S., Bhuiyan, J. and Besse, J.A. et al. 1999. Comparison of screening methods in the detection of bladder cancer. *J. Urol.*, 161:388–94.

Sanchez-Cespedes, M., Esteller, M. and Wu, L. et al. 2000. Gene promoter hypermethylation in tumors and serum of head and neck cancer patients. *Cancer Res.*, 60:892–5.

Sathyanarayana, U.G., Maruyama, R. and Padar, A. et al. 2004. Molecular detection of noninvasive and invasive bladder tumor tissues and exfoliated cells by aberrant promoter methylation of laminin-5 encoding genes. *Cancer Res.*, 64:1425–30.

Shen, L., Ahuja, N., Shen, Y. et al. 2002. DNA methylation and environmental exposures in human hepatocellular carcinoma. *J. Natl. Cancer Inst.*, 94:755–61.

Sherman, A.B., Koss, L.G. and Adams, S.E. 1984. Interobserver and intraobserver differences in the diagnosis of urothelial cells. Comparison with classification by computer. *Anal. Quant. Cytol.*, 6:112–20.

Sozen, S., Biri, H. and Sinik, Z. et al. 1999. Comparison of the nuclear matrix protein 22 with voided urine cytology and BTA stat test in the diagnosis of transitional cell carcinoma of the bladder. *Eur. Urol.*, 36:225–9.

Tada, Y., Wada, M. and Taguchi, K. et al. 2002. The association of death-associated protein kinase hypermethylation with early recurrence in superficial bladder cancers. *Cancer Res.*, 62:4048–53.

Valenzuela, M.T., Galisteo, R. and Zuluaga, A. et al. 2002. Assessing the use of p16 (INK4a) promoter gene methylation in serum for detection of bladder *cancer Eur. Urol.*, 42:622–8.

van Rhijn, B.W., van der Poel, H.G. and van der Kwast, T.H. 2005. Urine markers for bladder cancer surveillance: a systematic review. *Eur. Urol.*, 47:736–48.

Yates, D.R., Rehman, I. and Meuth, M. et al. 2006. Methylational urinalysis: a prospective study of bladder cancer patients and age stratified benign controls. *Oncogene*, 25:1984–8.