Can We Use Single Nucleotide Polymorphism and Runt Domain Transcription Factor 3 Methylation as Tumor Markers for Bladder Cancer?

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Purpose: Although many tumor markers have been evaluated in relation to bladder cancer, none of these biomarkers reported to date has shown sufficient sensitivity and specificity for the detection of the whole spectrum of bladder cancer diseases in routine clinical practice. The limited value of the established prognostic markers requires analysis of new molecular parameters of interest for predicting the prognosis of bladder cancer patients.

Materials and Methods: We conducted a review of the literature with a focus on recent advances in genetic polymorphism and hypermethylation events in relation to bladder transitional cell carcinoma.

Results: Recently, there has been major progress in both genetic polymorphism in relation to bladder cancer and molecular genetic and epigenetic changes leading to the development of transitional cell carcinoma. However, studies on numerous single-nucleotide polymorphisms in relation to bladder cancer have provided only a few genetic polymorphisms with only marginal information on patients' prognosis. For this reason, interest is increasing in epigenetic changes in bladder cancer. The epigenetic silencing of tumor suppressor genes is interesting from a clinical standpoint because of the possibility to reverse the epigenetic changes and thus restore gene function to a cell. Treatment with DNA methylation inhibitors can restore the activities of dormant genes and decrease the growth rate of cancer cells in a heritable fashion.

Conclusions: Epigenetic modification may be possible to partially reverse the cancer phenotype, and this will eventually lead to targeted therapy tailored toward specific molecular therapy in the near future. (Korean J Urol 2009;50:311-319)

Key Words: Urinary bladder neoplasms, Genetic epigenesis, Genetic polymorphism

INTRODUCTION

Bladder cancer consists of a broad spectrum of tumors that include transitional cell carcinomas, squamous cell carcinomas, and a few other tumor types. More than 90% of bladder cancers are transitional cell carcinomas and roughly 60% are low grade non-muscle invasive transitional cell carcinomas. The majority of these patients develop cancer recurrences after endoscopic resection, 16-25% with high-grade cancers. Approximately 10% of patients with non-muscle invasive bladder cancers subsequently develop invasive or metastatic disease. Almost 25% of patients with newly diagnosed bladder cancer have muscle-invasive disease, the vast majority being cancers of high histologic grade. Almost 50% of patients with muscle-invasive bladder cancer already have occult distant metastases.1-3

However, conventional histopathologic evaluation, encompassing cancer grade and stage, is inadequate to accurately predict the behavior of most bladder cancers. The need to establish which non-muscle invasive cancers will recur or
progress and which invasive cancers will metastasize has led to identification of a variety of potential prognostic markers for bladder cancer patients. Although precise reason why specific individuals get bladder cancer and progress to invasive disease with poor prognosis remains unknown, important determinants of population risk to bladder cancer may be mutation of proto-oncogenes and tumor suppressor genes, loss of heterozygosity for specific alleles, genetic polymorphism, and methylation patterns of promoter regions in specific genes.

Advances in molecular biology over the last decade succeeded in identification of many genetic alterations in bladder cancer. Despite the apparent de novo clinical presentation of invasive bladder cancers, cytogenetic, and antigenic evidence supports the hypothesis that transition cell carcinomas follow the general concept of multi-step carcinogenesis and proceed through two distinct genetic pathways responsible for generating different cancer morphologies. These are the inactivation of cyclin-dependent kinase inhibitors in low-grade transitional cell carcinoma and early p53-mediated abnormalities in high-grade transitional cell carcinoma. The progression of transitional cell carcinoma correlates with genetic instability and accumulation of collaborative genetic lesions.

Genetic variation in the human genome is an emerging resource to study cancer, a complex set of diseases characterized by both environmental and genetic contributions. It is becoming increasingly apparent that most of population-attributable cancer heritability is related not to the rare deleterious gene defects but to polymorphic variations in the DNA sequence. This concept has been substantiated in a variety of cancer settings, particularly by the genetic epidemiology of bladder cancer. Various enzymes, cytokines, and the gene repair system may be involved in carcinogenesis, recurrence, and progression of bladder cancer. Only a few genetic polymorphisms give us marginal information for patient’s prognosis, in spite of studies on numerous single nucleotide polymorphism (SNP) such as N-acetyltransferase 2 (NAT2), tumor necrosis factor-α (TNF-α), vascular endothelial growth factor (VEGF), glutathione S-transferase-α (GSTM1), glutathione S-transferase-θ (GSTT1), and human 8-oxoguanine DNA glycosylase 1 (hOGG1) in relation to the bladder cancer.

One of the few exceptions could be the association between the risk of bladder cancer and polymorphisms in two carcinogen-detoxification genes-NAT2 and GSTM1. Tobacco smoking is an important cause of bladder cancer, and previous analyses have suggested that the relative risk from smoking is stronger for NAT2 slow acetylators than for rapid or intermediate acetylators. This interaction is biologically plausible, since aromatic amines, which are thought to be the most important class of bladder
carcinogens in tobacco smoke,\textsuperscript{22} are detoxified by \textit{NAT2}.\textsuperscript{23} However, epidemiological evidence for this interaction is even weaker than for the overall genotype association. Several comparative studies among different ethnic groups observed less than 20\% and more than 55\% of slow acetylators among Asians and Whites, respectively.\textsuperscript{24,25} Among Korean subjects, 7.1\% of bladder cancer patients and 11.0\% of controls had slow acetylator genotypes.\textsuperscript{26} Yu et al\textsuperscript{27} observed that a difference in prevalence of slow acetylator (the high-risk phenotype) among the three ethnic groups (Whites, Blacks, and Asians) closely paralleled their varying incidence of bladder cancer. It might be inferred that the lower frequencies of slow acetylator genotypes in Asians relative to the Westerners could result in less incidence of bladder cancer.

\textit{GSTM1} null genotype has attracted much attention due to its possible association with increased susceptibility to certain malignancies such as lung cancer\textsuperscript{28} and bladder cancer,\textsuperscript{29,30} although the high risk of bladder cancer in individuals with the \textit{GSTM1} null genotype is still controversial. Kim et al\textsuperscript{26} demonstrated an association between the \textit{GSTM1} null genotype and increased risk of bladder cancer. These polymorphisms identified in GST and \textit{NAT2} genes, by modulation of mutagenic DNA adduct levels, may influence the occurrence and type of mutations in genes critical in tumor suppression (like \textit{p53}), thereby affect the individual susceptibility to cancer.\textsuperscript{31}

On the other hand, few studies showed \textit{GSTT1} being a possible risk factor in bladder cancer. Brockmoller et al\textsuperscript{32} reported no significant difference in the \textit{GSTT1} genotype between urothelial cancer cases and controls in all subjects including smokers and non-smokers, although a significantly high frequency of \textit{GSTT1} null genotype in the cancer cases was observed in the non-smoker group. To identify the effects of \textit{GSTM1}-null and \textit{GSTT1}-null types and smoking status on the bladder cancer incidence with ethnicity as determined by the previous 29 reports, Kim et al\textsuperscript{33} compared the frequencies of \textit{GSTM1}-null and \textit{GSTT1}-null genotypes and estimated smoking prevalence with the age-standardized bladder cancer incidence. In the univariate and multivariate analyses with the ecological data of various countries and ethnic groups, cigarette smoking positively, but the frequency of the \textit{GSTT1}-null type negatively, correlated with the age-standardized bladder cancer incidence. These results suggest that the \textit{GSTT1}-null genotype might not be a risk factor but a protective factor of bladder cancer.

Genes encoding a number of cytokines are polymorphic. \textit{TNF-\alpha} is a multifunctional cytokine. Genotype changes at position -308 of the \textit{TNF-\alpha} promoter are frequently observed in several cancers.\textsuperscript{34,35} \textit{VEGF} is a major angiogenic factor. \textit{VEGF} mRNA is markedly upregulated in human cancers, such as kidney and bladder cancer.\textsuperscript{36} \textit{VEGF} mRNA was found to be highly expressed in pT1 bladder cancer and to independently predict progression.\textsuperscript{37,38} Several studies reported that the cancer stage and grade were significantly associated with the \textit{TNF-\alpha} and \textit{VEGF} genotypes.\textsuperscript{39,40} Meanwhile, \textit{VEGF} expression did not correlate with cancer phenotype when assessed at the immunohistochemical level\textsuperscript{41-43} and further studies are warranted to better define its prognostic role in invasive bladder cancer.

DNA in most cells is regularly damaged by endogenous and exogenous mutagens. Unrepaired damage can result in apoptosis or may lead to unregulated cell growth and cancer. If DNA damage is recognized by cell machinery, several responses may occur to prevent replication in the presence of genetic errors. At the cellular level, checkpoints can be activated to arrest the cell cycle, and transcription can be up-regulated to compensate for the damage, or the cell can die. Alternately, the damage can be repaired at the DNA level enabling the cell to replicate as planned. Complex pathways involving numerous molecules have evolved to perform such repair. Because of the importance of maintaining genomic integrity in the general and specialized functions of cells as well as in the prevention of carcinogenesis, genes coding for DNA repair molecules have been proposed as candidate cancer-susceptibility genes.\textsuperscript{45-47} The mutagenic base, 8-oxoguanine, is removed from damaged DNA by base excision repair. \textit{hOGG1} gene encodes DNA glycosylase that catalyzes the excision of the mutagenic lesion 8-oxoguanine from oxidatively damaged DNA.\textsuperscript{48,49} A Ser326Cys polymorphism in \textit{hOGG1} has been identified in bladder cancers.\textsuperscript{40} Distribution of the \textit{hOGG1} codon 326 genotypes (Cys326Cys versus Ser326Ser and Ser326Cys) of controls was significantly different from the bladder cancer patients in that Cys326Cys genotype displayed a protective effect against bladder cancer development, compared to Ser326Ser and Ser326Cys genotypes. Currently, there is no convincing explanation that Cys326Cys is a highly protective genotype. These reflect that \textit{hOGG1} may not be the only gene associated with oxidative damage. An alternative DNA oxidative damage repair pathway to minimize the effects of 8-oxoguanine in genomes was reported.\textsuperscript{40}

Numerous SNP studies have been currently reported in
relation to the bladder cancer. The existence of low-penetrance cancer predisposing polymorphisms is undisputable; unfortunately, very few consistent gene-disease associations have been identified so far. It is hoped, that the ongoing worldwide efforts in obtaining large and informative DNA collections, combined with the rapid development of high-throughput genotyping technologies, will provide useful prognostic markers for clinicians applicable in clinical setting.

**EPIGENETIC ALTERATIONS**

The inheritance of information on the basis of gene expression levels is known as epigenetics, as opposed to genetics, which refers to information inherited on the basis of 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 methylation pattern is established during development and is normally 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. Enzymatic methylation of the C-5 position of cytosine residues can affect epigenetic inheritance by altering the expression of genes and by transmission of DNA methylation patterns through cell division. Cancer cells exhibit genome-wide hypomethylation accompanied by region-specific hypermethylation.\(^{31,52}\) DNA methylation may contribute to gene inactivation in cancer and therefore, DNA methylation is a powerful mechanism for the suppression of gene activity. The Rb gene was the first tumor suppressor gene for which hypermethylation was linked to tumorigenesis, and about 9% of retinoblastomas exhibit hypermethylation of the Rb 5’ region.\(^{53}\) Inactivation of gene expression by abnormal methylation of CpG islands can act as a “hit” for cancer generation.\(^{67}\) 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. In transitional cell carcinoma of the bladder hypermethylation of CpG islands around the promoter region and decreased expression of tumor suppressor genes, such as the RUNX3, p16 and E-cadherin genes, have been reported.\(^{5,54-56}\) Moreover, methylation pattern of RUNX3 promoter correlated strongly with transitional cell carcinoma of the urinary bladder.\(^{55}\)

Runt-related transcription factors are heterodimeric proteins characterized by a DNA-binding subunit and a non-DNA-binding β subunit. Runt domain transcription factors (RUNXs) are homologous to products encoded by the Drosophila segmentation genes runt and lozenge. They contain a conserved region termed the Runt domain, which is required for dimerization with a β subunit and for the recognition of cognate DNA-binding sequences.\(^{57}\) The N-terminal region of the runt domain contains 23 glutamine repeats followed by 17 alanine repeats. The C-terminal region is rich in proline-serine threonine, essential for transcriptional activation of target genes. All RUNX family members share the central Runt domain, which is well conserved and recognizes a specific DNA sequence, but each has relatively divergent N- and C-terminal regions.\(^{58}\)

The RUNX gene family consists of three members, RUNX1/AML1, RUNX2, and RUNX3.\(^{59}\) A single gene encodes the β subunit, CBF β/PEBP2 β.\(^{60,61}\) All three RUNX family members play pivotal roles in normal developmental processes and in carcinogenesis.\(^{59,62,63}\) The RUNX1 locus, required for definitive hematopoiesis, is the most frequent target of chromosome translocation in leukemia and is responsible for about 30% of the cases of human acute leukemia.\(^{64}\) RUNX2, essential for osteogenesis, is involved in the human disease cleidocranial dysplasia, an autosomal-dominant bone disorder.\(^{65}\) RUNX3 gene is located on human chromosome 1p36, a region that has long been suspected to harbor one or more suppressors of various cancers.\(^{66}\) Recent studies revealed that inactivation of RUNX3 due to DNA hypermethylation has been reported in various other cancers, including gastric cancer,\(^{67,68}\) lung cancer,\(^{69}\) hepatocellular carcinoma,\(^{70}\) breast cancer,\(^{71}\) colon cancer,\(^{72}\) pancreatic cancer,\(^{73}\) bile duct cancer,\(^{73}\) prostate cancer,\(^{73}\) larynx cancer,\(^{71}\) and bladder cancer.\(^{55}\) It is thus of interest to obtain more definitive evidence showing that RUNX3 is also a tumor suppressor in these cancers.

RUNX family transcription factors physically interact with TGF-β-activated Smads and mediate transforming growth factor β (TGF-β) signaling.\(^{74}\) Consistent with this scheme, members of the RUNX family share numerous important biological functions with members of the TGF-β superfamily,\(^{75}\) and RUNX proteins are therefore considered important for TGF-β signaling.\(^{76}\) RUNX3 activity is closely associated with TGF-β signaling since the gastric mucosa of the RUNX3
knockout mouse is less sensitive to TGF-β, inducing both cell cycle arrest and apoptosis. To inhibit the growth of a given cell type, TGF-β can employ diverse mechanisms, such as down-regulating c-myc and CDK-2/CDK-4 activity by modulating the functions of p15 INK4B, p21 WAF1/CIP1, and p27 KIP1. On the flip side, any genetic or epigenetic alteration of the TGF-β pathway can make normal cells vulnerable to tumorigenesis.51,62

Indeed, TGF-β receptors and SMADs are the main targets that are altered in numerous types of cancers, including pancreatic and colon cancers.53 One of the downstream targets of TGF-β is p21, the central role it plays in CDK inhibition and cell cycle control. The ability to modulate p21 expression and thus regulation of the cell cycle is often considered to be an intrinsic characteristic of many tumor suppressor proteins, including p53, BRAC1, and SMADs.63-67 RUNX3 is also an essential component of the TGF-β-mediated p21 induction that is critical for TGF-β-mediated cell growth inhibition. We revealed that RUNX3 is required for the TGF-β-dependent induction of p21 expression in stomach epithelial cells. Overexpression of RUNX3 potentiates TGF-β-dependent endogenous p21 induction. These findings suggest that at least part of the cancer suppressor activity of RUNX3 is associated with its ability to induce p21 expression.67

Although current pathologic and clinical variables provide important prognostic information, these variables still have limits for assessing the true malignant potential of most bladder cancers. Similarly, various diagnostic markers for bladder cancer development, recurrence, and progression have been reported, none adequate to predict the behavior of most cancers. RUNX3, as is also the case with other cancers, plays important roles in bladder cancer development, recurrence, and progression. Recently, we found significant effects of RUNX3 inactivation on the bladder cancer development, recurrence, and progression. RUNX3 methylation confers a 100-fold increase in the risk for bladder cancer development (OR, 107.55). RUNX3 methylation also seems 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 the aggressiveness of primary bladder cancers.55

However, using previously published data and newly added patients who underwent long term followup (median 50.0 months, range 1 to 121) with regular examinations, we noted that RUNX3 promoter hypermethylation is not significantly associated with bladder tumor recurrence.56 These inconsistencies may be due to the enormous array of clinical and pathological risk factors involved, such as the number of tumors, tumor size, prior recurrence rate, T category, carcinoma in situ, grade, intravesical therapy and other factors.89-91 These findings strongly suggest that inactivation of RUNX3 is causally associated with bladder cancer and that the methylation status of RUNX3 could be useful as a diagnostic marker for bladder cancer and as an indicator for bladder cancer and progression in the clinical setting.

The potential reversibility of DNA methylation patterns suggests that these are a viable target for the treatment of cancer. One specific goal of epigenetic therapy is to restore normal DNA methylation patterns and to prevent the cells from acquiring further methylation in DNA that could lead to silencing of genes crucial for normal cell function. Treatment of cancer cells with demethylating agents, such as 5-aza-2'-deoxycytidine, can reactivate a group of genes that are often crucial in controlling cell proliferation, differentiation, apoptosis and other key homeostatic mechanisms.62,95 Inactivation of DNA methyltransferases is the most effective way of inhibiting DNA methylation and re-establishing more normal patterns. Unlike many tumor suppressors, such as p53, which are inactivated mainly by deletions and mutations, RUNX3 is unique in that it is inactivated primarily by epigenetic silencing, rather than mutations or deletions. Furthermore, RUNX3 can be reactivated and therefore considered a good drug target because mutations in its gene are rare.94 Epigenetic drugs, such as DNA methylation inhibitors can restore the activities of RUNX3, target aberrantly heterochromatic regions, leading to reactivation of tumor-suppressor genes and/or other genes that are crucial for the normal functioning of cells. The use of these drugs can be therapeutic used alone or as part of a combination with other therapeutic modalities, such as chemotherapy, immunotherapy or radiotherapy. This will eventually lead to target therapy tailored toward specific molecular defects, thereby significantly lowering the morbidity associated with bladder cancer.

## CONCLUSION

Transitional cell carcinoma of the urinary bladder has a diverse collection of biologic and functional characteristics. Although, current pathologic and clinical variables provide important prognostic information, these variables still limits for
assessing the true malignant potential of most bladder cancers. Better understanding the molecular mechanisms involved in carcinogenesis and cancer progression has provided a large number of molecular markers of bladder cancer, with a potential diagnostic and prognostic value.

Numerous factors may be involved in carcinogenesis, progression and patient’s survival, including genetic polymorphisms, genetic and epigenetic alterations. Despite numerous SNP studies in relation to bladder cancer, only a few genetic polymorphisms give us marginal information for patient’s prognosis. The epigenetic silencing of tumor suppressor genes is interesting from a clinical standpoint since it is possible to reverse epigenetic changes and restore gene function onto a cell. It will be important to identify and elucidate the exact role of the key players involved in the generation of epigenetic patterns. 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. Reversing these epigenetic processes, restoring normal expression of malignancy-preventing-genes, has consequently become a new therapeutic target in cancer treatment. Aberrant patterns of epigenetic modification would be, in near future, crucial parameters in cancer diagnosis, prognosis and therapy while maintaining the quality of life.

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