Review Article

METHYLATION OF THE 5' CpG ISLANDS AND BLADDER CANCER PATHOGENESIS

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ABSTRACT

Deregulation of cell cycle that results in uncontrolled cellular proliferation is the basis of neoplastic process. Bladder tumors are heterogenous in their behavior, and it is very difficult to predict the clinical course in many patients. In order to alleviate this problem, attention has been focused on mutations in various cell cycle regulators and their association with tumor behavior. Mutations of the p16/CDKN2 gene, encoding a cyclin-dependent kinase inhibitor, are common in bladder cancer. In contrast to other tumor suppressor genes, the two most common mechanisms for loss of p16/CDKN2 function are homozygous deletion and loss of transcription associated with hypermethylation of the 5'CpG island region. Recently, it is found that methylation of p16/CDKN2 is potentially reversible with exposure to demethylating agents, such as 5-aza-2'-deoxycytidine, which is a well-established inhibitor of DNA methylation, which may open up new ways to effective tumor management.

Key Words: Methylation, Bladder cancer, Cell cycle

INTRODUCTION

In an attempt to identify the underlying mechanisms of neoplasia many studies have surveyed tumors and tumor cell lines for the presence of mutations in genes encoding cell cycle-related proteins (1). As a result of these studies, mutations in cell cycle genes constitute the most common genetic in tumor cells. In fact, almost all of the tumors have mutations in one of the genes involved in controlling progression trough the cell cycle. Malignant cells may acquire independence from regulatory signals that are normally required for a controlled cell cycle progression (2).

It has shown that DNA methylation is essential for normal embryonic development but alterations in DNA methylation are also very common in cancer cells and capable of directly modifying carcinogenesis. Current interest has focused on the potential of abnormal methylation events in silencing tumor suppressor genes, and causing their progressive epigenetic inactivation. Recent studies have shown that it is possible to reactivate these dormant genes by inhibitors of DNA methylation and potentially restore growth control of cells (3).

This review focuses on the importance of DNA methylation particularly in p16/CDKN2 in bladder carcinogenesis.

Cell Cycle, Cyclin-Dependent Kinases and Inhibitors

Cell cycle is a carefully programmed process in which just after division, the cell that is destined to divide again enters a stage called G1. Alternative is a resting phase called G0. A cell in G1 next moves into S phase, during which time DNA replication occurs, by a mechanism wherein each DNA strand serves as a template for its own replication. At the end of S phase, another gap phase (G2) begins, which then leads to actual mitosis (M). Extracellular signalling molecules, such as peptide growth factors, bind to specific cell-surface growth-factor receptors to modulate the cell cycle. Normal cells respond to these extracellular growth signals principally during the G1 phase. These factors induce resting cells (G0) to enter the cell cycle. However, some factors are necessary for the progression of cells from G1 phase into S phase (DNA synthesis). Once cells reach the G1/S transition and

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begin DNA synthesis, they become largely unresponsive to extracellular signals and proceed autonomously through DNA replication, G2, and mitosis.

Over the past few decades, studies by many groups have shown that passage of cells through the cell cycle depends on the activity of enzymes known as "cyclin-dependent kinases (Cdks)" a name indicating that they become active only when they associate with protein partners called cyclins.

Cdks, take place at the core of the cell cycle engine and drive cell proliferation forward by phosphorylating specific substrates in a cell cycle-dependent fashion. In order to become active kinases, the Cdks must associate with cyclins as well as undergo an activating phosphorylation. There are two types of primary G1 phase cyclins; D-type cyclins and cyclin E family (4). The D family of cyclins assemble into holoenzymes with the kinase catalytic subunits Cdk4 and Cdk6, while the principal partner of cyclin E is Cdk2.

G1 phase progression is also subject to negative regulation by a recently discovered group of molecules, the cyclin-dependent kinase inhibitors (CKIs). The mechanism whereby they achieve their function appears to be the formation of stable complexes that inactivate the catalytically operative units. Normal control of cellular growth requires a balance between the activators of cyclins and the inhibitors of the CDKs. Overactivity of the CDKs, whether by excessive production of cyclins or loss of CKIs, can result in aberrant growth of cells leading to cancer. CKIs family members can be subdivided into two groups on the basis of sequence homology. The first and probably best-characterized CKI family member to be identified was p21 (also known as WAFI, Cip1, or Sdi), which inactivates the cyclin E-Cdk2 complex, the cyclin A-Cdk2 complex, and the cyclin D1-, D2-, and D3-Cdk4 complexes (5). P21-WAF1 gene encodes a cyclin dependent kinase inhibitor and mediates tumor suppressor gene p53-induced cell cycle arrest. Overexpression of p21-WAF1 suppresses proliferation and growth of tumor cells in vitro, as well as in vivo (6). Another member of this group is p27/Kip1, which inactivates the same subset of cyclin-Cdk complexes as p21. The gene encoding p21 maps to chromosome 6p21.1, and the gene encoding p27 maps to chromosome 12p12-12p13. The other CKI subgroup includes three members: p16 (INK4A/MTS1/CDKN2) (7), p15(INK4B/MTS2) (4), and p18 (8).

The p16 gene has been found to be mutated frequently in a variety of human cancers. It is located at 9p21 and encodes a 16-kD protein; which is an inhibitor of cyclin D-Cdk4 and cyclin D-Cdk6 complexes required for Retinoblastoma protein phosphorylation (9,10). Loss of p16 by 9p21 deletion or mutation allows cyclin/CDK complexes to proceed with phosphorylation of Rb which inactivates this gene. In malignant cells, the cell cycle control pathway governed by the D-type cyclins is the one that is most commonly mutated in tumor cells. In normal cells, the positive effects of cyclin D compexes on cell cycle progression are counteracted by the p16/CDKN2 family of Cdk-inhibitor proteins.

Genetic Alterations and DNA Hypermethylation in Bladder Cancer

Molecular and cytogenetic abnormalities of 9p21 have been reported in several malignancies and tumor-derived cell lines, including melanoma (21), glioma (12), leukemia (13), lung cancer (14), head and neck squamous cell carcinoma (25), squamous cell carcinoma of bladder (16) and transitional cell carcinoma of the bladder (17). It has recently been demonstrated that the p16/CDKN2 cell cycle regulator gene may be inactivated by multiple mechanisms, including mutation, deletion, and methylation. Cytogenetic analysis of bladder cancer cells has revealed that they contain nonrandom choromosomal abnormalities, mainly monosomy of chromosome 9 (18). It was shown that loss of chromosome 9 is the most frequent alteration in urothelial transitional cell carcinoma (19).

It has previously been shown that allelic loss of chromosome 9 is possibly an early and common event in bladder carcinogenesis, and absence of blood group antigen expression is predictive in defining the tumor behavior (20). In the presence of chromosome 9 loss, tumor recurrence and/or progression was observed in 83% of bladder cancers. Molecular genetic studies of bladder tumors have shown further that LOH in chromosome 9 sequences is a frequent occurrence in papillary superficial tumors (21). Loss of heterozygosity on 9p21, where the p16/CDKN2 and the p15-INK4B cell cycle regulator genes are located, is a common genetic alteration in bladder cancer (22).

Orlow et al (23) reported an overall frequency of deletions and rearrangements for the p16 and p15 genes in bladder cancer of approximately 18% each. Their study did not detect any point mutations either in exon 2 of the p16 gene or in exons 1 and 2 of the p15 gene (75% of the total coding sequence). This study also revealed that the p15 gene and p16 gene, which are adjacent to one another, were almost always deleted together. Although, most of the p16/CDKN2 homozygous deletions also include the p15-INK4B locus, no intragenic mutations in p15-INK4B have

beed reported to date. Homozygous deletions have beed described to occur preferentially in low-grade bladder carcinoma. It should be emphasized that only Ta and T1, but not Tis, lesions showed deletions of either p16 or p15. In a recent study (24), it was demonstrated that p16 alterations occur independent of p53 mutations. Because p53 alterations commonly occur in Tis bladder tumors (25), it can be a hypothesis that bladder carcinogenesis might develop through two distinct molecular pathways (26).

The described tumor suppressor genes might be part of checkpoint pathways ensuring the accurate transmission of genetic information during cell division. In the case of inactivation of these genes by mutations, both promoting cell proliferation and accumulation of genomic errors lead to clonal evolution of cancer cells. Deletions at 9p21 have been reported in many human tumor types, including nearly 50% of bladder cancers (7). Several studies show homozygous deletions at gp21/CDKN2 in both uncultered and cultured bladder cancers (8), making CDKN2 a strong candidate tumor supressor gene.

DNA Hypermethylation

Recently, methylation, an epigenetic event, has been associated with the loss of CDKN2 expression in several cancers (27). DNA methylation in eukaryotic DNA is a normal postreplicative process and occurs at the 5^1-position of cytosine residues in the majority of CpG dinucleotides. This modification is associated with gene activity and is essential for normal mammalian development. Discrete regions of CpG-rich sequences withous methylation are clustered as CpG islands. These islands have been shown to be often associated with promoter regions of genes (28).

Alterations of DNA methylation patterns in these regions have important regulatory effects on gene expression. Hypermethylation of CpG islands have been shown to be associated with structural alterations in chromatin and transcriptional repression (29). CpG islands are commonly associated with housekeeping genes and may regulate their transcriptional activities. However, alterations in the methylation status of CpG islands during malignant transformation may be correlated with transcriptional changes in a number of genes associated with growth regulation, and it is possible that other genes involved in cell cycle control may also be influenced by these epigenetic mechanisms.

Approximately 1% of cytosines in vertebrate DNA are methylated at CpG dinucleotides (30). The presence of 5-methylcytosine at CpG dinucleotides may contribute to tumorigenesis either by generating point mutations or by altering gene expression (31). Deamination of 5-methylcytosine forms thymidine and generates a G-T mismatch which, if not reparied, further produces a A to U transition. A considerable variation also has been reported in mutational type at CpG sites among different types of cancer. Nevertheless, aberrant methylation of promoter regions resulting in inactivation of tumor suppressor gene expression has been proposed to be an important mechanism in cancer progression (32). In some tumors, methylation appears to supplement deletion or mutation, resulting in homozygous gene inactivation (33). The 5' region of CDKN2 contains numerous CG dinucleotides (termed "CpG island") that are unmethylated in the transcriptionally active gene (32).

The changes in DNA methylation that accompany carcinogenesis have been summarized in many studies. Decreases in the overall content of 5-mCyt (34), demethylation of specific loci (35), de novo methylation of CpG islands (36), and increased levels of DNA methyltransferase enzyme (37) have all been observed. These observations collectively have shown that methylation changes are highly consistent features of carciogenesis.

A variety of techniques have been previously developed that can be used to investigate patterns of DNA methylation, such as genomic sequencing (38), PCR-based methylation analysis (39), Southern blotting, and methylation sensitive AP-PCR (40). However, none of these can be used to isolate specific and unknown DNA sequences from genomic DNAs that are differentially methylated between normal and tumor tissues. Global changes in DNA methylation patterns are known to occur during tumorigenesis, and gene silencing has been associated with methylation of CpG islands located in, or near, promoters and 5' regulatory regions. With the exception of genes on the inactive X choromosome (41), Alu and L1 sequences (42), and some imprinted genes (43), CpG islands are usually unmethylated in normal somatic cells. In contrast, widespread methylation of CpG islans occurs on autosomal genes during oncogenic transformation. Aberrant methylation within 5' promoter region is associated with a transcriptional silencing of CDKN2 in many cancer cell lines as well as in lung, bladder, and colon tumors (27). This event is associated with a tightly compacted chromatin conformation around the CDKN2 promoter (29). Gonzalez et al (27) showed the methylation status and expression levels of the p16/CDKN2 tumor suppressor gene and the p15-INK4B cell cycle regulator in normal tissues, cell lines, and bladder and colon cancer; and they concluded that expression of the p16/CDKN2, but not expression of the p15 INK4B cell cycle regulator, is controlled by methylation of its 5' CpG island, and that de novo methylation of this island is a mechanism for

p16/CDKN2 inactivation in bladder cancer. They indicated that the 5' CpG island of the p16/CDKN2 TSG is frequently methylated in this tumor type. The high frequency (67%) of uncultered tumors showing de novo methylation of the p16/CDKN2 5' CpG island could explain the low rate at which p16 homozygous deletions and intragenic mutations are found in bladder cancer.

They pointed out that, in contrast to squamous cell carcinoma of the bladder in which p16 is frequently deleted (15), de novo methylation of the 5' CpG island of p16/CDKN2 may be a more common mechanism of inactivation of this tumor suppressor gene in transitional cell carcinoma (TCC). They also showed that the methylation of the 5' CpG island of p16/CDKN2 occurred not only in bladder tumors and bladder cell lines but also in normal colon mucosa.

Jarrard et al (44), showed that 14% of primary prostate tumors demonstrated aberrant methylation. They pointed to the absence of p16/CDKN2 methylation in normal, BPH, and seminal vesicle tissues suggesting that methylation is an event associated with neoplastic transformation. Recent evidence actually demonstrates that abnormal hypermethylation of CpG islands exists in a variety of human neoplasia, commonly in solid tumors, including transitional cell carcinoma of the bladder (67%), breast carcinoma (31%), glioma (24%), and colorectal carcinoma (10-40%). Some studies also showed that hypermethylation of the p16/CDKN2 promoter region is a frequent and early occurring event during the process of neoplastic progression in ulcerative colitis. (45). In this study Hsieh et al. pointed out that p16/CDKN2 promoter methylation is a candidate biomarker in the surveillance of patients with ulcerative colitis and p16/CDKN2 repression by promoter methylation has a major role in the process of neoplastic progression in this group of patients.

DNA Hypermethylation Inhibition

There are several features of the abnormal methylation patterns in tumor cells making them attractive targets for therapeutic intervention. The growth-regulating genes are present in a suppressed form, that makes it theoretically possible to restore expression and hence reinstate growth control. In addition to this, genes silenced by methylation errors are very sensitive to reactivation by inhibitors of DNA methylation such as azacytosine nucleosides (30).

The epigenetic inactivation of p16/CDKN2 gene expression is potentially reversible with exposure to demethylating agents, such as 5-aza-2'-deoxycytidine, which is a well-established inhibitor of DNA methylation (46). This agent, inhibits 5-methyltransferase, and induces CDKN2 reexpression in several tumor types, including bladder cell lines containing aberrant methylation. Castello et al (29) demonstrated that this reexpression is associated with a relaxation of tightly compacted chromatin in the CDKN promoter region. Bender et al. (3) showed that after exposing seven human tumor cell lines and two human fibroblast cell strains to the demethylating agent, 5-aza-2'-deoxycytidine (5-Aza-CdR), to determine whether the silencing of growth-regulatory genes by de novo methylation in immortalized cell lines could be reversed, possibly restoring growth control, this agent suppressed cellular growth in all seven tumor lines but not in fibroblast strains. They concluded that 5-Aza-CdR may slow the growth of tumor cells by reactivating growth-regulatory genes silenced by de novo methylation.

Bender et al. also reported that p16 induction and growth suppression by 5-Aza-CdR treatment are heritable and dose dependent, supporting findings by Castello et al. in which 5-Aza-CdR mediated p16/CDKN2 expression was heritable in vitro. Jarrard et al. in an attempt to evaluate the effect of this demethylating agent on transcription, treated PC3, PPC1, and TSU-PR1, cell lines with dense 5' CpG island methylation, for 5 days. Reexpression of the p16/CDKN2 transcript by RT-PCR, and a partial demethylation of the 5' CpG island in Southern analysis, morphologic alterations in culture consisting of cellular aggregates formation, and decreased growth rate were noted in this study (47).

Herman et al. (33), at the basis of the association of hypermethylation in the 5' promoter region with lack of transcription of the normal mRNA, examined cell lines of breast, prostate, and colon cancer for p16/CDKN2 expression by RT-PCR. They demonstrated that no methylated cell line expressed the expected p16 product. In contrast, normal female breast tissue and three of four tested normal colon samples had detectable p16 message. To confirm that DNA methylation blocked transcription, they treated the colon cancer cell lines with the demethylating agent, ant they noticed that both colon cancer cell lines had detectable p (p16) 16 mRNA after treatment with 5-Aza-CdR. And also they pointed that the aberrant DNA methylation is essential for maintaining transcriptional silencing. All these results suggest cancer therapies in which growth-regulatory genes (like p16) silenced by de novo methylation are reactived by 5-Aza-CdR, effectively suppressing the growth of human tumor cells.

However, according to Jones (47) in spite of their potency and specifity for inhibition of DNA methylation, the chemical instability of 5-aza-nucleosides in

aqueous solution and present suspicion about the carciogenecity and mutagenecity of 5-Aza-CdR make it unlikely that these drugs will find widespread clinical use. Therefore, there is a need to search for other inhibitors of the DNA methyltransferase.

Conclusion

As the genes and pathways governing cell cycle control are better understood, their involvement in human cancer becomes clearer. How these molecules interact with one another to control normal cell proliferation and role of their loss in contribution to tumorigenesis have not been understood exactly, and may be the most important question will be whether they constitute useful therapeutic targets in cancer cells. Changes in the DNA methylation machinery and alterations in DNA methylation patterns and levels are very common in cancer cells, including bladder cancer. A highly consistent finding in cancer cells is that the CpG islands of growth-regulatory genes are often methylated, in contrast to the situation in their normal counterparts. These changes could play a direct role in the induction of point mutations that inactivate tumor suppressor genes. This may also be an important aspect of future therapeutic approaches.

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