REVIEW

The emerging role of epigenetics in urological cancers

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DNA methylation and histone modifications constitute the common epigenetic modifications in vertebrate genomes. The epigenetic changes are early event in the cancer development and are reversible. Over the last decade, the field of epigenetics has made considerable progress both in the diagnosis and treatment of variety of malignancies. Novel epigenetic markers are being studied, which have the potential as sensitive diagnostic and prognostic markers. DNA methylation has been identified as a powerful diagnostic tool in classification, detection and risk assessment of cancers. As DNA methylation is reversible, inhibitors of DNA methyl transferases and histone deacteylases have been designed for use in treatment of a variety of urological malignancies. Variety of drugs targeting epigenetic changes are being studied, which can be effective individually or in combination with other conventional drugs used in cancer therapy. The emerging area of epigenetic therapy holds great promise for novel chemotherapeutic and chemoprevention approaches against cancer.

Key Words: epigenetics, DNA methylation, urological cancers

Introduction

Epigenetics is the study of stable, yet reversible alterations in gene expression that occur during development and cell proliferation. Epigenetic changes do not modify the actual genetic sequences. These modifications, together with the promoter sequences and transcription factors modulate gene expression. Epigenetics can also be explained as a phenomenon arising from the interplay between environment and genetics.[1-4] Recent studies have shown that epigenetics plays an important role in cancer biology, somatic gene therapy, viral infections and genomic imprinting. Epigenetic changes, particularly the DNA methylation is found to be involved in a variety of cancers including colon, lung, breast and ovarian cancers apart from prostate cancer. Unlike passively transferred genetic

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mutations, the epigenetic changes must be actively maintained and its "reversibility" makes them a potential therapeutic target.[3] In this review, we discuss the basics of epigenetics and their potential diagnostic and therapeutic applications in urological malignancies.

Basics of epigenetics

Genome

Approximately 23,000 genes are contained in the human genome. For proper functioning of the cells, these genes should be expressed in specific cells at specific times.[5] The chromatin is a nucleoprotein complex made of nucleosomes. The nucleosomes are made of DNA which are wrapped around octamers of globular histone proteins.[6] The changes in the chromatin structure influence the gene expression. When the chromatin is condensed, the gene expression is "switched off" and when it is open, the gene expression is "switched on".[7] The status of chromatin is dynamic and can be controlled by reversible epigenetic mechanisms.

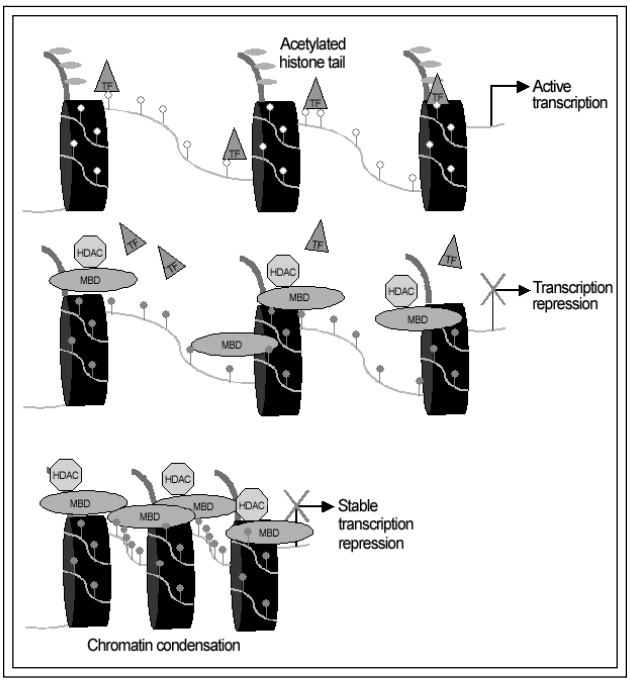

Figure 1. Epigenetic silencing of gene expression. The promoter of an actively transcribed DNA has unmethylated CpGs (white circles) and is associated with acetylated histones. This facilitates an open configuration that enables association of transcription factors (TFs) to the promoter sequence. DNA methyltransferases transfer methyl groups to CpG dinucleotides (methylated CpGs are indicated by shaded circles) that occlude transcription factor binding resulting in transcriptional repression. Methyl binding domain (MBD) proteins then bind to methylated CpGs, and recruit histone deacetylases (HDAC), which deacetylate histones leading to chromatin compaction and stable repression of transcription.

The two important, well-studied epigenetic mechanisms are DNA methylation and histone modifications such as acetylation. These two processes can act independently and/ or together affecting the gene expression and in turn the tumorigenesis. Figure 1 shows a schematic overview of epigenetic silencing of gene expression.

DNA methylation

DNA methylation occurs by transfer of methyl group by DNA methyl transferases (DNMTs) that target cytosine residues in 5′-CpG 3′ dinucleotides. Cytosine methylation is the commonly occurring epigenetic event that regulates gene expression in mammals. DNA methylation occurs in the normal genome and 70%-80% of CpG dinucleotides are heavily methylated in human cells. But CpG islands comprising of stretches of CG rich DNA (approximately 1 kb) are protected from methylation. Methylation of these normally unmethylated CpG islands and the subsequent loss of gene expression is a major event in initiation of cancer.[8] However it is still unclear why protection of CpG islands from methylation is lost in cancer cells. Aging, chronic inflammation and viral infections have been identified as causes of DNA methylation. Recent studies have proposed the presence of CpG island methylator phenotype (CIMP), in which increased methylation of CpG islands was observed. But this hypothesis is disputed.[9]

There are four well characterized DNMTs in human cells- DNMT1, DNMT2, DNMT3a and DNMT3b.[10] Over expression of DNMTs has been observed in several cancers and increased DNMT1 expression in normal cells leads to *de novo* CpG methylation.[11,12] Transcript levels of DNMTs are differentially regulated during cell cycle and alterations in mRNA levels of DNMT may contribute to aberrant methylation, which is typically observed in cancer cells. Knock down of DNMT1 gene resulted in 80% loss of promoter methylation and re-expression of otherwise silenced tumor suppressor genes.[13] RNAi mediated knockdown of DNMT1 also resulted in reduced cell viability.[14] The enzymes by themselves have also been shown to repress gene transcription. A C/T polymorphism in the DNA methyltransferase 3b (DNMT3b) promoter region results in increased activity and have been identified as a risk factor for lung cancer.[15] Our studies on prostate cancer

suggested that DNMT3b polymorphisms may be associated with an increase in promoter methylation of tumor-suppressor genes related to the development of prostate cancer, and may thereby increase the risk of this disease.[16]

Methyl binding domain proteins

Methyl group attached to cytosine presents steric hindrance to the binding of transcription factors, thereby repressing the transcription machinery. The predominant mechanism of methylation mediated transcriptional repression occurs by the binding of transcriptional repressor proteins either directly by the methyl binding domain (MBD) or indirectly by interaction with other proteins that bind to methylated DNA. Thus far, six methyl-binding proteins have been described. MBD1, MBD2, MBD3, MeCP2, and KAISO are all involved in transcriptional repression.[3] MBD4 has been an exception since it was long thought to be a DNA repair protein. However, recently, it has been demonstrated that MBD4 has the ability to repress transcription of p16 and hMLH1 genes through binding to methylated CpG.[17] MBD1, MBD2, MBD3 and MeCP2 share a highly conserved MBD domain. The MBD is sufficient to direct specific binding to methylated DNA.[18] Regions outside the MBD contribute to overall binding energy through non-specific, presumably electrostatic interactions.[19] A second functional domain, the transcriptional repression domain or TRD, is required for transcriptional repression.[20] KAISO, on the other hand, contains a transcriptional repressor domain but lacks an MBD. KAISO binds to methylated CpGs via a zinc finger domain.[21]

Histone modifications

Eukaryotic DNA is organized in the form of an extensively folded package of DNA-protein complex referred to as chromatin. Histone proteins form an important part of this nucleosome complex and play an important role in regulation of gene expression. Acetylation of lysine residues at N-terminus of histones removes positive charges, thus loosening the binding between DNA and histones. This allows easier access of RNA polymerase and transcription factors to the regulatory regions and enhances transcription of the gene. Histone deacetylation on the other hand results in tight wrapping of DNA around the histones leading to repression of transcription. Histone acetylation and deacetylation are catalyzed by histone acetylase transferases (HATs) and histone deacetylases (HDACs) respectively. Histone methylation by specific histone methylases also plays a role in chromatin remodeling and gene regulation.[3] Methylation of histone H3 at lysine 9 is associated with deacetylation of H3 and hypermethylated trsncriptionally inactive hMLH1 promoter whereas methylation of histone at lysine 4 is associated with acetylated H3 and unmethylated gene promoter.[22] Since MBDs target histone deacetylase complexes to methylated DNA, it was believed earlier that histone modification was the result of DNA methylation. However recent studies provide evidence that histone modifications can cause DNA methylation.[3,23]

Epigenetic markers in urological cancers

Over the past few years, better understanding of chromatin structure, DNA methylation, histone modification and transcription activity have resulted in an integrated view of epigenetics. Aberrant methylation and silencing of tumor suppressor genes have been documented as a common occurrence in cancer. This has led to vast application of this information in cancer diagnosis, prognosis and therapeutics.

Diagnostic techniques

The epigenetic state of a gene is assessed by (1) measuring gene expression at transcript and protein levels, (2) determining histone modifications and chromatin composition and (3) analyzing promoter DNA methylation status.[23] Gene expression levels are measured by microarray analysis that has provided a powerful tool in predicting clinical outcome or response to therapy. Chromatin immunoprecipitation precipitation has been used in studying chromatin composition and histone modifications.[24] However, this technique has not yet evolved as a clinically useful diagnostic method. Techniques used for analysis of DNA methylation status include sodium bisulfite conversion, CpG island microarrays and mass-spectrometry.[25] After bisulfite treatment unmethylated cytosines are converted to uracils while methylated cytosines are unchanged. Bisulfite treated DNA is then analyzed by sequencing, methylation specific polymerase reaction (MS-PCR), combined bisulfite restriction analysis, methylation sensitive single nucleotide primer extension (MS-SNuPE) and methylation sensitive single strand conformational polymorphism. Primers for bisulfite treated DNA are designed using software programs available for the purpose. MS-PCR is the most useful method for detection of DNA methylation since it is quick, easy and reproducible. The CpG microarray and the differential methylation hybridization (DMH) methylation assay are validated for examining multiple

TABLE 1. **Common epigenetic markers in urological cancers**

Tumor	Methylation markers	Samples used for analysis	References
Prostate	GSTP1, TIG1, APC, RARβ2, RASSF1A, TMS1, CDH13	Tissue, serum, urine	3,27,30,45
Bladder	DAPK, BCL2, TERT, RASSF1A, EDNRB, TNFRSF25	Tissue, urine, serum	31
Kidney	P16INK4α, p14ARF, VHL, APC, MGMT, RARβ2, GSTP1, RASSF1A, ECAD, Timp3	Tissue, urine, serum	32

CGI loci at a time. The high-throughput nature of microarray studies permits analysis of methylation profile of tumors, which would in turn help in determining the response to chemotherapeutic agents and survival.[25]

DNA methylation as biomarker

DNA methylation is an early event in cancer and hence is a potent molecular marker in early detection of the disease. Biomarkers are useful not only for the early detection of malignancies but also for monitoring of recurrence of cancer and as a prognostic tool. Several studies have shown the presence of tumor derived free DNA in the serum, plasma or urine of cancer patients.[3] Aberrant DNA methylation in the serum/plasma appears to be a specific marker for various types of cancers including prostate, bladder and kidney cancers.[26-29] The genes that are aberrantly methylated in urological malignancies are listed in Table 1.

Promoter hypermethylation of the glutathione S-transferase P1 gene (GSTP1) is the most frequent DNA alteration in prostatic carcinoma and occurs very early in the onset of the disease. GSTP1 methylation is present in prostate cancer and high-grade prostatic intraepithelial lesions (PIN), but absent in normal prostatic tissues. Hence, GSTP1 hypermethylation has been used as the most common biomarker in early detection of prostate cancer in body fluid samples.[30] In this study, 39% of patients with prostate cancer had detectable GSTP1 methylation (58% sensitivity). However, GSTP1 methylation was also detected in 33% of patients without evidence of cancer in biopsy. Other genes that are commonly methylated in prostate cancer include Tazarotene-induced gene 1 (TIG1), adenomatous polyposis coli (APC), retinoic acid receptor β2 (RARβ2), Ras association domain family 1A (RASSF1A), target of methylation-induced silencing (TMS1) and H-cadherin 13 (CDH13).[3] Genes commonly methylated in bladder cancer include death-associated protein kinase (DAPK), B-cell leukemia/lymphoma 2 (BCL2), Telomerase Reverse Transcriptase (TERT), RASSFIA, endothelin receptor type B (EDNRB) and tumor necrosis factor receptor superfamily, member 25 TNFRSF25.[31] Significant increase in methylation of these genes was observed in bladder cancer tissues compared to adjacent non-malignant tissues ($p \leq 0.01$). Analysis of urine sediments for gene methylation showed methylation of DAPK, BCL2 and TERT in 78% of the bladder cancer cases. No methylation of these genes was detected in urine from age matched healthy controls (100% specificity).[31] Potential biomarkers for kidney cancer include the INK4 proteins (p16 INK4a and p14ARF), Von-Hippel Lindau gene (VHL), APC, O-6-methylguanine-DNA methyltransferase (MGMT), GSTP1, RARb2, RASSF1A, E-cadherin (ECAD), and Tissue Inhibitor of Metalloproteinases-3 (Timp-3).[32] Hypermethylation of at least one gene was found in all the tumor tissue DNA analyzed. These results matched with methylation pattern in urine samples in 88% of the cases. No methylation of the genes was detected in normal kidney tissue or urine from healthy individuals or individuals with benign kidney disease (100% specificity). Analysis of multiple gene methylation patterns might provide a more accurate and sensitive early detection of cancer.

Epigenetic therapy

The reversibility of DNA methylation makes it a potential therapeutic target for treatment of cancer. In vitro experiments have shown that demethylating drugs result in reactivation of otherwise silenced genes. These drugs cause inhibition of DNMT or HDAC activity and reverse the process of epigenetic silencing. Substrate analogues such as 5-azacytidine (Vidaza) and 2-aza-deoxycytidine (Decitabine) have

been used as inhibitors of DNMTs.[33] Both these drugs have obtained FDA approval for use in cancer therapy. These compounds are incorporated into DNA and trap DNMT. The trapped DNMT is then degraded and demethylation is induced.[34] Apart from demethylating effects, 5- azacytidine nucleotides are shown to possess cytotoxic properties also. However, the cytotoxic effects were considerably reduced when DNMT1 was knocked down, hence providing a direct link between the inhibitory activity and toxicity of the compounds. These drugs have also been shown to activate transcription of genes with unmethylated promoters and also cause increased acetylation and histone H3 lysine 4 methylation. Both "5-Azacytidine" and "2-aza-deoxycytidine" have been used in phase I, II and III trials with success,[35,36] however the major limitation in the use of these drugs in cancer treatment is the lack of specificity and stability. This has led to the search for alternative approaches to inhibit DNMTs. Small interfering RNAs (siRNAs) have been used to achieve targeted knockdown of DNMTs and are currently being tested in phase II trials for their effectiveness in tumor therapy. DNMT1 knockdown has been reported to cause significant demethylation and reactivation of several epigenetically silenced genes. However the therapeutic use of siRNA in therapy has remained disputed since DNMT1 may be dispensable for maintenance of epigenetic changes in cancer cells. Moreover the delivery problems of siRNA under in vivo conditions have not yet been resolved.[37]

Recently additional DNMT inhibitors have also been discovered. Zeularine, a stable derivative of azacytidine has attracted considerable interest in epigenetic therapy.[38] This compound was originally synthesized as a cytidine deaminase inhibitor but has been shown to possess DNMT inhibitory activity. Besides, the drug can be orally administered and has shown to cause demethylation and reactivation of epigenetically silenced genes. Zebularine is minimally cytotoxic and can be administered continuously at a low dose to maintain demethylation for a prolonged period of time.[39] Procaine and procainamide have been reported to have demethylating properties. These compounds do not become incorporated into DNA but act by binding to CpG rich sequences. This blocks the binding of DNMTs to their target sequences and results in DNA demethylation. Another molecule shown to cause demethylation is epigallocatechin-3-gallate (EGCG), which is the main polyphenol compound in green tea. In vitro treatment of cancer cells with this compound has shown to cause reduced DNA methylation and increase in transcription of tumor suppressor genes. However, EGCG influences a wide variety of cellular processes and direct inhibition of DNMTs is yet to be demonstrated. Another class of DNMT inhibitors includes psammaplins, which target both DNMT and HDAC activity resulting in combinatorial loss of both the epigenetic pathways. But psammaplins target other enzymes involved in DNA metabolism and lack of specificity limits the use of this drug in cancer therapy.[37] Functional characterization of human DNMTs will provide essential information for designing novel pharmacological inhibitors of epigenetic targets with improved specificities.

Deacetylation of histones by HDACs is commonly associated with epigenetic silencing. A number of histone deacetylase inhibitors (HDACi) have been developed as another class of drugs for epigenetic cancer therapy, and first-generation drugs are currently being tested in phase I/II clinical trials. There is increasing knowledge about the role of HDACs in tumorigenesis and the action of HDACi. HDACis induce differentiation, growth arrest and apoptosis both in vitro and in vivo. Accumulation of acetylated histones leads to induction of silenced genes. The commonly used HDACis include phenyl butyric acid,[40] suberoylanilide hydroxamic acid (SAHA),[41] depsipeptide[42] and valproate.[43] Due to the existence of many different HDACs, there is need to design targeted HDACs that target individual enzymes, thus enhancing the specificity of this treatment.

Since epigenetic mechanism is a complex process involving a number of pathways, combination therapy would be the best strategy to achieve maximum treatment efficacy. Combinations of DNMT inhibitors and HDAC inhibitors have been shown to produce a synergistic effect in activation of silenced genes. Further, cytotoxicity can be reduced by using combinations of DNMT inhibitors. An initial dose of decitabine followed by an extended treatment with zebularine has been shown to induce long-term demethylation.[44] These results suggest that novel combinations of epigenetic therapeutic drugs need to be studied to achieve stable and effective DNA demethylation.[43]

Conclusions

Epigenetic changes in urological malignancies are being studied extensively at present and genome wide screening will lead to development of novel epigenetic markers. There is increasing knowledge on the

molecular mechanisms of epigenetic silencing. Epigenetic changes are early event in cancer development and hence can be used to assess the risk of developing cancer. Therapeutic drugs that reverse these epigenetic changes have the potential to be an effective adjunct treatment for variety of malignancies. However, they need to be studied both for its efficacy and safety profile. Gene specific epigenetic drugs need to be developed for better targeting of the disease. Unfolding the epigenome would answer many biological and medical questions on the initiation and progression of cancer. □

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