Molecular Markers for Diagnosis, Staging, and Prognosis of Bladder Cancer

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By Stephen G. Williams, MD [2], Maurizio Buscarini, MD [3], and John P. Stein, MD, FACS [4]

Conventional histopathologic evaluation of bladder cancer, encompassing tumor grade and stage, is inadequate to accurately predict the behavior of most bladder tumors. Intense research efforts are under way to identify and

transitional cell carcinoma of the bladder, the second most common tumor of the genitourinary tract, is also the second most common cause of death from these cancers. In 2001, an estimated 54,300 new cases will be diagnosed in the United States, and some 12,400 patients will die of the disease.[1]

Approximately 80% of patients with primary bladder cancer experience low-grade tumors confined to the superficial mucosa, and the majority are amenable to initial transurethral resection and selected administration of intravesical immunotherapy or chemotherapy.[2] The risk of recurrence for patients with superficial bladder tumors can be as high as 70%, however. One-third of recurrent tumors may demonstrate tumor progression to a higher grade and/or stage of disease. Muscle-invasive tumors occur initially in 15% to 30% of all bladder cancer patients; 50% of those treated locally for invasive tumors will relapse with metastatic disease within 2 years of treatment.[3] These data underscore the heterogeneous nature and malignant potential of transitional cell carcinoma of the bladder.

The optimal management of invasive bladder cancer requires the detection and accurate assessment of the tumor’s biological potential. Treatment strategies for patients with bladder cancer are currently dictated by histologic evaluation, including determination of tumor grade and stage as the primary prognostic variables. Although these two conventional histopathologic variables provide a certain degree of stratification of a tumor’s biological characteristics, there remains a significant degree of tumor heterogeneity within the various prognostic subgroups. This makes it difficult to accurately and reliably predict the tumor’s aggressiveness. The ability to precisely predict an individual tumor’s true biological potential would facilitate treatment selection for patients who may benefit from adjuvant therapy and identify patients who may require less aggressive strategies.

Intense research efforts are ongoing to best characterize bladder cancer and its varying biological profiles.

Transitional cell carcinoma of the bladder has generally been viewed as two different disease processes. Superficial tumors are most often locally proliferative and recurrent but can become invasive and even metastatic. Superficial tumors that maintain a malignant phenotype may be treated more effectively with early, aggressive intravesical therapy or even cystectomy. The use of molecular markers may guide decision-making in the treatment of superficial bladder cancers.[4]

Muscle-invasive cancer, notorious for its potential clinical virulence, is ideally treated aggressively,[2] but there remains a significant incidence of recurrence and disease progression in some patients, who may ultimately benefit from an adjuvant form of therapy.

The need to predict which superficial tumors will recur or progress[and which invasive tumors will metastasize] has led to an ongoing search for improved understanding of bladder carcinogenesis and metastasis. With the advent of new molecular techniques, the field of medical molecular biology has exploded in recent years, resulting in detailed analysis of human cells and tissues on the DNA, RNA, and protein levels. The molecular and genetic changes in transitional cell carcinoma of the bladder can be schematically classified into three separate, but intertwined, events: (1) chromosomal alterations, representing the initial event in carcinogenesis; (2) tumor proliferation, caused by loss of cell-cycle regulation; and (3) metastasis, in which the initial tumor breaks from its original confined environment, aided, in part, by processes such as angiogenesis and loss of cell adhesion.

We believe that the accumulation of these successive genetic alterations[rather than a single genetic event in time] determines a tumor’s phenotype and, subsequently, the patient’s clinical outcome. In this review, we will summarize the recent literature regarding molecular and genetic
changes in bladder cancer and comment on potentially improved diagnostic tools and treatment regimens becoming available as a result of our better understanding of these molecular pathways.

**Expanding Diagnostic Modalities**

Our understanding of tumor biology has evolved rapidly over the past decade. Advances in molecular biology, immunology, and cytogenetics have prompted this progress. An increase in knowledge has provided an opportunity to identify and evaluate tumor characteristics beyond the scope of general histology and gross DNA content and to distinguish the potential behavior of an individual tumor. As the role of tumor markers in the diagnosis and prognosis of bladder cancer grows, it is important to understand the various technologic, methodologic, and analytic issues regarding each diagnostic modality. Several techniques can be applied to evaluate a tumor marker; most are only applied in a research setting, however.

For the clinical urologist, the most widely used evaluation method is immunohistochemistry. Translational research has enhanced the application and evaluation of immunohistochemical techniques with potentially important clinical roles in bladder cancer. Currently, most tumor markers that have been studied and merit a role in the contemporary clinical decision-making process for bladder cancer have evolved from the application of immunohistochemistry. Standardization of the technique and its interpretation will ultimately be necessary for successful and consistent application of immunohistochemistry. With proper controls and standardization, immunohistochemistry will remain one of the principle techniques used to evaluate various tumor markers. Although efforts have been made to standardize immunohistochemistry, the limitations of this technique must be realized before we can develop a consensus as to how to perform the procedure. Despite these limitations, immunohistochemistry maintains some translational application in the analysis of various bladder cancer markers. Other more sophisticated and costly techniques, such as single-strand conformational polymorphism, DNA sequencing, or polymerase chain reaction (PCR)-based analyses, are currently used in the research setting but are not yet applicable to clinical decision-making.

**Carcinogenesis of Bladder Tumors**

**Oncogenes**

Bladder cancer is an excellent model for the study of molecular changes at the DNA level, owing to its distinctly different subtypes (superficial and muscle-invasive) and their different propensities to progress. Such DNA alterations in bladder cancer have been studied in a variety of ways, ranging from cytogenetics to DNA ploidy to loss of heterozygosity.[5] DNA alterations can result from a number of genetic insults such as point and insertional/deletional mutations, translocations, and loss of alleles. Each insult may affect the translated protein product. The large fund of molecular knowledge developed in recent years about carcinogenesis has provided some evidence about the different genetic pathways for bladder cancer.

Earlier work in the field of molecular oncology focused on oncogenes, which are normal cellular genes that contribute to the malignant phenotype of a tumor by overexpressing the normal gene product or, in some cases, by expressing a protein product with altered function. Overexpression of the normal gene product is usually achieved by gene amplification or chromosomal translocation of the gene to an area downstream of a powerful promoter. However, expression of a mutated protein product can also lead to activation of the malignant phenotype. Oncogenes believed to be important in human malignancies include c-H-ras, c-myc, MDM2, and HER2/neu (aka c-erbB2).

**c-H-ras Gene** The c-H-ras gene is an active oncogene thought to be involved in the development and progression of human bladder cancer. Mutational studies of the ras gene family have demonstrated that alterations in codons 12 and 61 of the H-ras gene occur in up to 20% of bladder cancers.[6-8] One study, employing PCR amplification followed by oligonucleotide-specific hybridization, reported that 36% of bladder tumors had the same mutation on codon 12 of the H-ras gene.[9] In general, the activation of H-ras occurs by a single-point mutation (G → A) in codon 12, although other mutations have been described.[6]

 Clinically, Fontana and colleagues demonstrated a statistically significant relationship between the overexpression of the c-ras oncogene and early recurrence in patients with superficial bladder cancer.[10] These data suggest a potential prognostic role for the c-ras oncogene in patients with superficial bladder cancer, but currently these techniques apply only in a research setting.

**c-myc Gene** An important regulator of cellular proliferation, the myc gene family encodes for nuclear phosphoproteins containing DNA-binding activity.[11] The c-myc oncogene has been shown
to be overexpressed in several human tumors including bladder cancer. Deregulation of the \textit{myc} gene family occurs with chromosomal translocation and gene amplification, and studies have demonstrated that \textit{myc} overexpression promotes cellular proliferation. Although the genetic mechanism causing overexpression of the \textit{c-myc} gene in bladder cancer is unknown, its overexpression has been shown to be associated with high-grade bladder cancer. Kotake and associates demonstrated that expression of the \textit{c-myc} gene product correlates with the nuclear grade of bladder cancer. In a study with conflicting results, however, Lipponen found no independent prognostic value for Myc proteins with respect to prognosis for patients with transitional cell carcinoma of the bladder. Currently, the prognostic significance of \textit{c-myc} gene expression is unknown, and further evaluation will be required to determine its prognostic role.

\textit{HER2/neu} The proto-oncogene \textit{HER2/neu} has been extensively studied and implicated in a number of tumors, including breast, prostate, and bladder cancers. The \textit{HER2/neu} oncogene encodes a transmembrane glycoprotein similar to the epidermal growth factor (EGF) receptor, having tyrosine kinase activity and the ability to stimulate cellular growth. Initial studies of \textit{HER2/neu} were performed in breast carcinoma and demonstrated a significant relationship between gene expression, tumor progression, and overall survival. Subsequently, several studies reported that \textit{HER2/neu} expression in bladder cancer patients is associated with higher-stage tumors, increased tumor progression, greater incidence of metastasis, and reduced overall survival. These investigations suggest a prognostic value of \textit{HER2/neu} expression in human bladder cancer; other studies have reported conflicting results, concluding that evaluation of the oncogene provides no additional prognostic value over the previously established predictors of grade and stage for transitional cell carcinoma. In light of these discrepant results, further evaluation will be required to accurately determine the prognostic value of \textit{HER2/neu} in bladder cancer.

\textbf{Tumor-Suppressor Genes}

Recent work in the field of chromosomal alterations has focused on identifying specific loci on chromosomes that may contain altered genes. Many of these genes have been identified as tumor-suppressor genes that, when inactivated, result in initiation and/or progression of the malignant phenotype. Recently, with the advent of such techniques as loss-of-heterozygosity analysis and comparative genomic hybridization, a significant increase in genome scanning has occurred and identified many new chromosomal alterations in transitional cell carcinoma. Loss-of-heterozygosity analysis employs known polymorphic markers to identify large deletions and/or alterations of both alleles of a chromosome. In the most common scenario, one large deletion of an entire chromosome results from natural genetic recombination and is followed by a small alteration in the retained allele involving an insertion/deletion or point mutation, usually at a specific locus containing a tumor-suppressor gene. Comparative genomic hybridization utilizes genomic DNA from tumor and normal cells that is differentially labeled by fluorescence. The two DNA extracts are then hybridized onto a platform of the normal metaphase spreads of all human chromosomes. A loss or amplification of a particular DNA sequence on the tumor DNA is determined by comparing label intensity with the normal hybridized DNA.

Using these molecular techniques, an extensive search for tumor-suppressor genes in recent years has led to the discovery of several key genes on various chromosomes. Deletions on the short arms of chromosomes 3 (3p) and 8 (8p) have been detected in high-grade, muscle-invasive bladder cancer. In fact, the 8p deletion has been noted in more than 50% of muscle-invasive transitional cell carcinomas, a number similar to the 42% rate of \textit{p53} (aka TP53) mutations in stage T2 to T4 bladder tumors. No deletions were noted in superficial transitional cell carcinoma. Loss of heterozygosity on the short arm of chromosome 8 (8p) has been associated with high grades and stages of transitional cell carcinoma. However, the relevant genes have not yet been identified. The most notable chromosomal deletions in bladder cancer have been detected on chromosomes 9, 13, and 17. This has led to the identification of the retinoblastoma (Rb) gene on chromosome 13, the \textit{p53} gene on chromosome 17, and promising new tumor-suppressor genes on chromosome 9 at the p21 locus. In fact, deletions of chromosome 9 are the most common chromosomal abnormalities, found in more than half of all grades and stages of transitional cell carcinoma. Although most muscle-invasive bladder tumors have other chromosomal alterations as well, most Ta and T1 tumors show few genetic alterations other than those on chromosome 9.

\textit{Chromosome 9} The majority of chromosome 9 deletions are located on the short arm at 9p. Specifically, a complex genomic region at 9p21 (INK4a/ARF and INK4b) encodes three distinct
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proteins—p16, p14ARF, and p15—all of which act as negative cell-cycle regulators and are considered potential tumor-suppressor genes.[31] Extensive screening of the retained allele in bladder tumors with 9p loss of heterozygosity has not revealed frequent mutations. Instead, homozygous deletions of both alleles appear to be the common mechanisms for inactivating the entire locus in transitional cell carcinoma.[32-34] Such a deletion will commonly remove all three 9p21 genes. While the clinical implications of such homozygous deletions have not been evaluated, in vitro transfection studies using bladder cancer cell lines have demonstrated growth arrest following the introduction of p16 into cell lines with deleted INK4.[35] The p14ARF, p15, and p16 proteins are currently under investigation as markers of malignant potential; this topic will be discussed below in the section on cell-cycle regulation.

Chromosome 13
The Rb gene was the first tumor-suppressor gene to be isolated. It codes for a nuclear protein mapping to 13q14.[36] Like p53, Rb is thought to play an important role in bladder cancer progression. Rb gene mutations are noted in 25% to 30% of bladder tumors,[37,38] and loss of heterozygosity at the Rb locus (13q) is strongly associated by immunohistochemistry with an absence of Rb protein expression.[39]
Unlike p53, missense mutations are rare in retinoblastoma and no mutational hotspots have been identified. In fact, mutations are distributed throughout 24 of the 27 exons in hereditary retinoblastoma. The large size of the gene does not lend itself to standard DNA sequencing or single-strand conformational polymorphism analysis[40]; therefore, detailed analyses of specific mutations within the Rb gene are unlikely to be used in clinical decision-making.

Chromosome 17
Two other important chromosomal alterations that affect known tumor-suppressor genes involve 17p13 (the site of the p53 gene) and 13q14 (the site of the Rb gene). The importance of p53 in bladder cancer was suggested by the high frequency of loss of heterozygosity for chromosome 17p in high-grade transitional cell carcinoma.[41] Other studies have confirmed that p53 follows classic tumor-suppressor theory, with loss of heterozygosity at the 17p allele and mutation of the remaining Tp53 allele. This pattern has subsequently been recognized in a large number of muscle-invasive bladder tumors, but with lower frequency in superficial tumors.[42,43] These genetic defects have been shown to correspond with protein expression of the mutated p53 gene product.[44,45]
The evaluation of p53 is a good example of the difficulty of making the transition from genetic studies to translational research techniques like immunohistochemistry. Nearly 25% of tumors that are p53-positive by immunohistochemistry show no detectable mutations by standard gene sequencing analyses, while about 10% of wild-type tumors (p53-negative) harbor readily identifiable mutations.[45,46] Still, p53 remains the best characterized tumor-suppressor gene and is clearly implicated in disease progression in several solid tumors, including bladder cancer.

p21 Locus
In a recently reported study, 12 of 19 bladder tumor cell lines had identifiable mutations in the central core domain of exons 5 to 11, with concomitant loss of Rb protein expression. The other seven bladder cancer cell lines studied showed normal wild-type p53 or mutations only in exons 1 to 4, but all seven had concomitant alterations at the 9p21 (INK4a/ARF and INK4b) gene locus.[47] This study provided the first evidence of possible differences in the penetrance of p53 mutations in bladder cancer, while adding evidence to the concept of multiple genetic pathways of bladder carcinogenesis first proposed by Spruck and colleagues.[48] Although we are gaining a better understanding of the actual molecular events leading to the initiation and progression of bladder cancer, none of the techniques currently available to identify allelic instability or loss of heterozygosity are yet applicable for clinical use. The advent of high-throughput molecular technology, however, may allow for clinical testing of specific chromosomal alterations in individual patients in the near future.

Cell-Cycle Regulatory Pathways
Normal cellular proliferation occurs in an orderly progression through the cell cycle, which is regulated by cell-cycle-associated protein complexes composed of cyclins and cyclin-dependent kinases (Cdks).[49] Several tumor-suppressor genes acting at the G0/G1 checkpoint of the cell cycle are now recognized, and their protein products (p53, pRb, p16, and p14) are vital to preventing cell-cycle progression in bladder tumors. Inactivation of one or more tumor-suppressor genes and loss of cell-cycle control appear to be early steps in the development of carcinogenesis and, ultimately, cancer progression.
Inactivation of a gene can occur by mutation, deletion, or methylation and, in most cases, requires alteration of both copies of the gene. The case of p53 is an exception, however, since alteration of
only one copy of the gene is sufficient to alter function. The inactivation of both copies of a gene can occur by one of two pathways: either the primary inherited alteration of one copy followed by a second "hit" occurring somatically (ie, caused by environmental mutagen exposure or dysfunction of DNA replication/repair), or entirely somatic events in which two independent "hits" occur in both gene copies.[46]

Tumor-Suppressor Genes
Most of the initial studies on cell-cycle regulation in bladder cancer focused on the roles of individual tumor-suppressor genes and their protein products. More recent investigations have identified many pathways within the cell cycle. These pathways involve the interaction of several tumor-suppressor genes, and it is this interaction that is most likely responsible for bladder cancer progression.

**pRb Gene**

The pRb gene is located on chromosome 13q14 and encodes for a 110-kD nuclear phosphoprotein.[50] Although initially discovered to be mutated in patients with inherited retinoblastoma, altered Rb gene expression has been reported in various human tumors, including transitional cell carcinoma of the bladder.[37,38,51] In its physiologic active hypophosphorylated form, pRb acts by inhibiting cell-cycle progression at the G1/S checkpoint. However, pRb interacts with various cell-cycle regulatory proteins. These proteins include cyclins, which catalyze inactivation of pRb via phosphorylation; Cdk inhibitors (p21, p16, p27), which inactivate Cdk/cyclin complexes and thus inhibit pRb phosphorylation; and the E2F family of transcription factors, which are responsible for transactivating genes necessary for entry into the S phase of the cell cycle.[52] Any alteration in these interactions can lead to uncontrolled cell growth.

Inactivation of the Rb gene is thought to be an important step in bladder cancer progression. With a combination of immunohistochemical techniques and molecular analysis, several groups have demonstrated that the proportion of tumors demonstrating Rb alterations increases with higher-grade and -stage bladder cancers.[39,53] The results of these studies suggest that loss of pRb expression may be an important prognostic factor in transitional cell carcinoma of the bladder. Cordon-Cardo and associates report that patients with muscle-invasive bladder tumors who have lost Rb immunoexpression have a significantly shorter 5-year survival than patients with normal Rb protein expression.[38] Similarly, Logothetis and colleagues demonstrated that Rb alterations were more common in advanced tumors, and that patients who had lost pRb expression experienced shorter overall survival compared with patients who maintained Rb expression.[37] Based on the data, it appears that pRb expression is an important prognostic factor in patients with invasive bladder cancer.

**p53 Gene**

Mutations in the p53 gene are the most common genetic defect in human tumors.[54] The p53 gene is located on chromosome 17p13, encodes for a 53-kD protein, and is known to play a vital role in the regulation of the cell cycle.[55] When DNA damage occurs, the level of p53 protein increases, causing cell-cycle arrest. This allows for the repair of DNA and prevents propagation of the DNA defect. Mutations in the p53 gene result in the production of an abnormal and usually dysfunctional protein product with a prolonged half-life compared to the wild-type protein. Consequently, this abnormal protein accumulates in the cell nucleus and can be detected by immunohistochemical staining. Several studies have demonstrated that nuclear accumulation of p53 protein determined by immunohistochemical staining correlates with gene mutations detected by DNA-sequence analysis.[56,57,45]

Alteration of p53, as determined by immunohistochemical techniques, is an important prognostic indicator for bladder cancer progression.[44,58,59] Increased p53 immunoreactivity appears in higher-grade and stage bladder cancers, and is associated with disease progression and reduced overall and disease-specific survival. Our group evaluated p53 nuclear reactivity in 243 patients with invasive bladder cancer who were uniformly treated by radical cystectomy.[58] Patients with altered p53 and increased p53 expression experienced a significantly greater risk of disease recurrence and reduced overall survival compared to patients without altered p53. This association was strongest in patients with organ-confined bladder tumors (pathologic stage P1, P2, or P3a). Furthermore, nuclear accumulation of p53 was determined to be the only independent predictor of disease progression in a multivariable analysis of p53 status, histologic grade, and pathologic stage. Accumulating evidence suggests that the mutation status of p53 varies greatly, and that not all p53 mutations affect the cell cycle in the same manner. Most studies of p53 mutations have identified central core alterations in exons 5 to 8.[3] The frequency of mutations outside this region (exons 2 to 4, and 9 to 11) is lower, although still highest in bladder cancer compared with other cancers.[60] In bladder cancer cell lines, Markl and Jones have shown that mutations in exon 4 were always associated with loss of p14 and p16; mutations in exons 5 to 11 were always paired with loss of pRb.[47]
Combination of Rb and p53 Genes

Two independent studies have evaluated the prognostic significance of combining the Rb and p53 status of bladder cancers as determined by immunohistochemical techniques.[61,62] Preliminary data from these studies support the concept that bladder tumors with alterations in both p53 and Rb are associated with a poorer prognosis and reduced overall survival compared with tumors with wild-type p53 and wild-type Rb. Tumors with an alteration of only one of these genes behave in an intermediate fashion. These data suggest that the status of both p53 and Rb are important, and that these two proteins act in an independent, yet synergistic manner, in patients with bladder cancer.

Role of p21 Expression

In fact, p53 functions as a cell-cycle regulatory protein[57] and mediates its effects on the cell cycle, in part, through the regulation of p21WAF1/CIP1 expression.[49] Alterations in p53 can result in loss of p21 expression, leading to unregulated cell growth. However, not all p53-altered bladder tumors recur or progress.[58,59] It has recently been demonstrated that p21 expression may also be mediated through p53-independent pathways.[63,64] This important finding suggests that despite the presence of a p53 alteration, p21 expression and, therefore, cell-cycle control can be maintained.

We found that immunohistochemical detection of p21 protein in the nuclei of bladder cancers showing p53 alterations provides important additional prognostic information for patients. Tumors from 101 patients who underwent radical cystectomy for invasive bladder cancer were evaluated for p21 expression.[64] All of the patients had been previously determined to have p53-altered tumors.[58] Patients who were p21-negative demonstrated a significantly increased probability of recurrence and a significantly decreased probability of overall survival, compared with patients whose tumors maintained p21 expression. The association between p21 status and prognosis in p53-altered bladder tumors was independent of tumor grade, pathologic stage, and lymph node status.

Loss of p21 expression was strongly associated with a higher probability of recurrence and longer survival in patients with lymph node-negative organ-confined disease and lymph node-negative extravesical disease. These findings suggest that p21 expression through p53-independent pathways exists, and that cell-cycle control may be maintained through these pathways. Patients with p53-altered tumors that lose p21 expression appear to have a poor prognosis and may be managed best with aggressive forms of therapy.

Loss of Cell-Cycle Control

The interaction of p53 and p21 in cell-cycle regulation and the cooperative effects of p53 and Rb are good examples of the mounting evidence that mutation in a single tumor-suppressor gene is unlikely to be the only factor resulting in carcinogenesis. We now understand that there are several pathways within the cell cycle and that each of them plays a role in cell-cycle regulation. Alteration of one or more of these pathways is likely responsible for bladder cancer progression.

Cyclin-Dependent Kinases

The 9p21 locus is a complex region of chromosome 9, as discussed previously, where many deletions have been identified in bladder tumors and bladder cancer cell lines. This locus (ie, p21, p16, and p27) has proven crucial in the regulation of the cell cycle because of the unusual situation whereby the two functionally different genes, p16 and p14, are both transcribed from the same locus, but via alternative first exons and reader frames.

A well-characterized Cdk inhibitor,[65] p16 (aka INK4a, Mts1, CDKN2A) functions upstream of pRb to block cyclin-D-directed phosphorylation of Rb, thus inducing G1 arrest. Mutations and homozygous deletions of p16 are common in bladder cancer cell lines, in squamous cell carcinoma, and bilharziasis-associated bladder cancers.[52] Furthermore, p16 is thought to be susceptible to transcriptional silencing by promoter methylation.[66] Inactivation of p16 by any of the mechanisms will lead to uninhibited phosphorylation of pRb and subsequent cell proliferation.

The other gene product at 9p21 is p14 (aka ARF, murine p19). Acting upstream of p53 to stimulate p21 expression, p14 may also play an important role in the feedback loop that regulates the cellular level of p53 by interacting with the cellular proto-oncogene product MDM2.[67] However, p14 is very different from p16 in that it is expressed ubiquitously, whereas p16 expression is more restricted. Moreover, p14 does not bind to cyclin-dependent kinases or function as a Cdk inhibitor,[68] and it can cause cell-cycle arrest at any point in the cycle through its effect on p21.[46]

Whereas the majority of research on the 9p21 locus has been performed in vitro using cell lines and animal models, Orlow and colleagues recently examined deletions of the INK4a gene in 121 patients with superficial (Ta or T1) bladder tumors. They found that homozygous deletions of the INK4a gene resulted in lower recurrence-free survival. Furthermore, deletions that affected both p16 and p14 by deregulating both the p53 and pRb pathways correlated with larger and higher-grade tumors.[69] As previously mentioned, p14 regulation of the cell cycle can occur through physical interaction with...
MDM2.[70] In normal cells, MDM2 regulates p53 function by marking p53 for degradation via ubiquitin conjugation and inactivates p53 by binding to its transactivation domain. However, p14 binding of MDM2 appears to counteract these effects by protecting p53 from degradation.[71] Nevertheless, the role of MDM2 in regulating p53 protein levels in transitional carcinoma of the bladder remains unclear. The frequency of MDM2 gene amplification across all malignancies is thought to be uncommon (~7%), with the highest (20%) frequency in soft-tissue tumors. MDM2 gene amplification is infrequent in bladder cancer, with one study showing MDM2 amplification in only 1 of 87 cases, despite elevated MDM2 protein levels in 26 of the cases.[72] MDM2 amplifications and p53 mutations usually do not occur within the same tumor sample, indicating that carcinogenesis can result from MDM2 amplification alone. Thus, the role of tumor-suppressor genes in cell-cycle regulation is a complex one, with the 9p21 gene locus lying at the center of p53 and Rb, the two major tumor-suppressor pathways identified in bladder cancer.[1] Figure 1 summarizes the two pathways and the various tumor-suppressor gene interactions described above.

Angiogenesis

Angiogenesis, the formation of new blood vessels from the surrounding established vasculature, is a tightly regulated, essential physiologic process that occurs during normal development, reproduction, and repair. Uncontrolled angiogenesis can lead to a variety of pathologic states and participates in the maintenance of neoplastic conditions. In its simplest form, angiogenesis can be described in three steps: (1) initiation and activation of the endothelial cell; (2) migration and invasion of the activated endothelial cell after proteolytic degradation of the surrounding extracellular matrix; and (3) maturation of the endothelial cells to coalesce and form watertight tubules that establish blood flow.[73] Understanding this complex process in an attempt to inhibit tumor angiogenesis has been the focus of expanding interest and investigation in the field of oncology because of its potential therapeutic benefits. Stimulation of angiogenesis, or neovascularization, is a critical adaptation characteristic of all solid tumors. Without angiogenesis, tumor growth is inhibited at a diameter of 2 to 3 mm—the natural limit for diffusion of essential nutrients and oxygen.[74] Under most homeostatic conditions, new blood vessel formation is infrequent and is controlled by an abundance of inhibitory signals directed at the endothelium that sets the balance in favor of vascular quiescence. It is thought that the overall balance of stimulatory and inhibitory inputs to the endothelial cells determines the angiogenic phenotype for any given tumor. A multitude of inducers and inhibitors have been identified in solid tumors. During disease states such as carcinogenesis, the angiogenic balance within the tumor’s microenvironment shifts in favor of endothelial cell activation. Folkman has termed this the "angiogenic switch": New vessel growth is stimulated, thus providing the necessary nutrients for continued tumor growth and eventual metastasis.[75] Current research has identified several mechanisms by which this angiogenic switch can occur, including overexpression of inducers and/or loss of endogenous inhibitor production.[76] These factors may be produced by the tumor cells themselves or released from the surrounding extracellular matrix and tumor-associated stromal cells, or they may be products of inflammatory cells that infiltrate the tumor.[77,78]

Microvessel Density

As both tumor growth and invasion depend, in part, on this angiogenic response, the ability to quantitate the degree of angiogenesis within or around a given tumor may provide prognostic information. This has been accomplished by determining the so-called "microvessel density" within and around a given tumor using antibodies to factor VIII and CD34 that recognize immature or new vascular endothelial cells. Microvessel density has been demonstrated to be a useful prognostic indicator in a variety of malignancies including melanoma,[79] breast cancer,[80] and prostate cancer.[81] In general, increased microvessel density counts have been associated with tumor progression and survival reduction.[81,82] The relationship between microvessel density count and tumor progression has also been examined in patients with bladder cancer.[83,84] Dickinson and colleagues evaluated a series of 45 patients with invasive bladder tumors and, with a median follow-up of 37 months, found the microvessel density count to be an independent prognostic indicator of disease progression. Patients with an elevated microvessel density count demonstrated a 2.5-fold greater risk of dying.[85] Our group recently evaluated the relationship between tumor angiogenesis and tumor progression in
164 patients with invasive bladder cancer.[83] In this study, microvessel density was significantly associated with both disease recurrence and overall survival in these patients following radical cystectomy. Patients with elevated microvessel density counts demonstrated a significantly increased risk of disease recurrence and a worse overall survival, compared to patients with low microvessel density counts. Furthermore, microvessel density count was determined to be an independent prognostic indicator of both disease progression and overall survival when evaluated in the presence of histologic grade, pathologic stage, and regional lymph node involvement.

Angiogenic Inducers

It is difficult to evaluate the angiogenic potential of any given tumor because of the abundance of proangiogenic factors that are produced by tumor cells or released by the surrounding extracellular matrix (Table 1).[8,86-99] Prevailing evidence for the balance hypothesis proposed by Folkman suggests that an angiogenesis suppressor gene encodes or controls expression of one or several angiogenesis inhibitors that maintain a quiescent vasculature in cells. The theory maintains that the angiogenic inhibitor is downregulated during tumorigenesis, shifting the balance in favor of the angiogenic inducers with subsequent endothelial cell proliferation and migration; hence, the term "angiogenic switch."[75]

The angiogenic properties of urine from patients with transitional cell carcinoma were first noted by Chodak and associates, who documented a stimulatory effect on the migration of endothelial cells exposed to the urine.[99] Basic fibroblast growth factor (bFGF), a potent proangiogenic factor, has been shown to be excreted in high levels in the urine of bladder cancer patients, compared with patients without evidence of disease.[88,100] Urinary bFGF has also been correlated with the pathologic stage of the primary tumor in patients with muscle-invasive tumor.[89]

Recently, elevated levels of vascular endothelial growth factor (VEGF), another important angiogenic inducer, have been discovered in the urine of bladder cancer patients. Crew and colleagues evaluated 98 patients and showed VEGF levels to be highest in the urine of patients with bladder cancer, compared to normal controls and patients with other unrelated malignant conditions. In addition, they found that VEGF levels correlated with tumor recurrence in patients with Ta and T1 disease.[101]

We recently identified elevated levels of VEGF in the urine of bladder cancer patients with high-grade and/or muscle-invasive transitional cell carcinoma, compared with prostate cancer patients and patients without evidence of malignancy. In 92 patients undergoing radical cystectomy, higher VEGF levels in urine obtained preoperatively were associated with significantly reduced 3-year survival.[102]

While numerous proangiogenic factors have been identified in bladder cancer cell lines and tissues, Campbell and colleagues have shown that VEGF and bFGF appear to be two primary inducers of angiogenesis in bladder cancer cell lines.[90] Neutralizing antibodies to VEGF and, to a lesser extent, to bFGF significantly reduced the angiogenic activity of bladder cancer cell lines; neutralizing antibodies to acidic fibroblast growth factor, scatter factor, transforming growth factor-alpha and -beta, and thymidine phosphorylase did not. O'Brien and colleagues provided data on how tissue levels of VEGF taken from human bladder cancer tumors correlate with stage progression in superficial transitional cell carcinoma. They found a fourfold increase in VEGF mRNA levels in Ta tumors, compared with normal urothelium, and a 10-fold increase in T1 tumors. T1 tumors also had increased expression of VEGF mRNA, compared with invasive tumors.[91]

Other researchers have confirmed similar expression of VEGF in normal urothelial tissue and in bladder cancer. Campbell and associates identified relatively constant levels of VEGF immunostaining across normal urothelium and in superficial and muscle-invasive bladder cancer.[90] Sato et al found the VEGF transcript present in both normal urothelium and bladder cancer tissue. However, muscle-invasive tumors expressed significantly higher levels of VEGF by Northern blot analysis.[103]

Angiogenic factors appear to be produced directly by the bladder cancer cells. The immunostaining pattern for bFGF appears to be unique: bFGF localizes primarily to the basement membrane rather than tumor cells. O'Brien and colleagues have hypothesized that tumor-induced degradation of the basement membrane could release bFGF, accounting for the increased levels in the serum and urine of bladder cancer patients.[104]

Angiogenesis Inhibitors

Much of the research to date in bladder cancer angiogenesis has focused on inducers. Because of the balance hypothesis, we know that this represents only part of the puzzle of predicting a tumor's metastatic potential. Many inhibitors of angiogenesis exist, including thalidomide (Thalomid).[105]
interleukin-12 (rhIL-12),[106] angiostatin,[107] and thrombospondin-1 (TSP-1).[108,109] Only TSP-1 has been examined in human bladder cancer, although angiostatin inhibited the growth of Lewis lung carcinoma,[107] and human breast,[110] colon,[110] and prostate[111] cancers in animal models. Thrombospondin-1 is an extracellular matrix glycoprotein that is a potent inhibitor of angiogenesis both in vitro and in vivo.[112,113] Campbell and colleagues demonstrated that conditioned medium from normal urothelial cells contained high levels of TSP-1 and could inhibit angiogenesis induced by VEGF and bFGF in bladder cancer cell lines. Furthermore, they showed that the inhibitory activity of TSP-1 could be relieved by neutralizing antibody.[90] Our group reported that TSP expression can be determined using antigen retrieval immunohistochemistry in routinely processed formalin-fixed, paraffin-embedded tissue.[114] Employing this technique, we evaluated 163 patients with invasive bladder cancer for TSP expression. Patients with low TSP expression exhibited higher recurrence rates and shorter overall survival, compared with patients with moderate- or high-TSP expression. This association was strongest in patients with organ-confined disease. Furthermore, TSP expression remained an independent predictor of both disease recurrence and overall survival in the presence of tumor stage, histologic grade, and lymph node status. In this same cohort of patients, we also found that tumors with low TSP expression were significantly more likely to demonstrate high microvessel density counts.[115]

**Extracellular Matrix and Metastasis**

The ability of a tumor to invade surrounding stroma is one hallmark of metastasis. Stromal/epithelial interactions and matrix-degrading enzymes undoubtedly play a role in the tumor’s ability to invade. The composition of the extracellular matrix serves to maintain endothelial cell function, and it provides scaffolding for endothelium attachment and for migration during capillary formation. Joseph and colleagues have shown that bladder cancer cells can induce the production of the angiogenesis inducer scatter factor by the underlying stromal cells.[116] Matrix metalloproteinases are also thought to play an important role in degradation of the extracellular matrix. The matrix metalloproteinases MMP-2 and MMP-9 are elevated in the serum and urine of patients with muscle-invasive bladder cancer and correlate with poorer disease-free survival.[97] MMP-9 has increased expression in transitional cell carcinoma compared to normal urothelium, and also correlates with increasing tumor stage.[117]

**Antiangiogenic Therapy**

As a result of the improved understanding of the activated vessels associated with neovascularization of solid tumors, clinical trials have now begun for some solid tumors in an attempt to develop effective antiangiogenic therapies. Targeting tumor angiogenesis provides several advantages over conventional forms of treatment. First, blood vessels are readily accessible via the circulation and are required for all tumor growth and metastasis, yet are not necessary for normal physiologic function except for wound-healing and female fertility.[118] Second, the endothelium targeted by an antiangiogenic approach is nonneoplastic and maintains its full complement of regulatory mechanisms. Endothelial cells are unlikely to undergo the changes that result in drug resistance in solid tumors.[119] Furthermore, there is a paucity of side effects associated with antiangiogenic therapy. The lack of bone marrow suppression or gastrointestinal mucosal alteration[120] makes it a more desirable form of treatment than conventional chemotherapy. Anti-VEGF therapy, consisting of humanized monoclonal antibodies directed at the VEGF protein, has demonstrated antitumor activity in animals.[121] All hereditary, and most sporadic (clear cell) renal cell carcinomas, are associated with a defect in the VHL tumor-suppressor gene on chromosome 3p. A function of the VHL gene regulates the expression of proteins, including the suppression of VEGF. VEGF overexpression occurs after a mutation in the gene or a partial deletion of chromosome 3, and is thought to contribute to the many vascular manifestations characteristic of VHL syndrome.[122] Anti-VEGF therapy is now under investigation as a novel therapy against metastatic renal cell carcinoma.[123]

A clinical trial using antiangiogenic agents in bladder cancer has yet to be conducted, but its potential benefits are obvious. Antiangiogenic therapy could be used for chemoprevention in patients at high risk for recurrence or progression, possibly through intravesical administration. Adjuvant therapy for patients at high risk for progression (ie, advanced tumor stage, regional nodal involvement, and altered p53) following radical cystectomy would be another indication. This approach is particularly appealing, since the tumor burden is relatively low. Experimental models suggest that maintaining micrometastases in a dormant state for prolonged periods may be
reasonable goal, especially for older patients. Younger patients would likely require long-term or intermittent administration of antiangiogenic agents to maintain disease-free or disease-stable states.[124]

There is now ample evidence to suggest that bladder cancer is, in part, dependent on angiogenesis for growth and metastasis. However, angiogenesis remains a complex and tightly coordinated process that is not yet fully understood. Recent work has suggested that upregulation of inducers such as bFGF and VEGF, and/or downregulation of inhibitors like TSP-1 are important in determining a tumor’s angiogenic phenotype. We are probably seeing only the tip of the iceberg in understanding how complex cellular interactions lead to tumor progression and metastasis. Although therapies directed at individual inducers hold promise, the complexity of the angiogenic process makes it unlikely that targeting a single inducer will be adequate treatment. Nevertheless, improved understanding of the molecular pathways regulating angiogenesis will undoubtedly improve tumor prognostication and treatment options.

**Future Directions**

The translational application of various tumor markers for bladder cancer to both prognostic evaluation and therapeutic treatment plans continues to evolve. The ideal tumor marker should possess a degree of sensitivity and specificity approaching 100%. An ideal marker should also provide predictive information of the disease’s natural history, its response to treatment, and its likelihood of recurrence or progression. This marker should provide staging information complementary to that obtained from imaging studies or by invasive methods. It should also provide information for the follow-up of patients. Finally, it must have technical characteristics that allow standardization and reproducibility.[125]

After nearly a decade of intense study from laboratories around the world, we are not yet able to reliably apply any marker to clinical practice. Great strides have been made in efforts to develop and apply prognostic markers to this disease, however. We are now beginning to witness the emergence of molecular markers that may prove useful in the management of patients with both superficial and muscle-invasive bladder tumors.

The application of markers by urologists and oncologists to clinical decision-making will require strict standardization of techniques and interpretation of the results. Future use depends upon development of automated systems that can reliably evaluate tumor markers tested by both immunohistochemical and molecular techniques. This technology will help to eliminate or reduce inter- and intraobserver variability. Furthermore, these automated systems may reduce costs and personal manpower requirements, providing quicker and more consistent results.

**Overall Strategies**

The uncontrolled cellular proliferation that leads to invasive and metastatic capabilities is a complex process, and the measurable determinants of these properties most likely will not involve a single marker. A considerable amount of redundancy is inherent in all biological systems, and the evaluation of one particular marker will not logically guarantee the behavior of the tissue. The ultimate application of tumor markers may involve the evaluation of numerous tumor markers for an individual tumor or specimen. Simultaneous evaluation of these tumor markers using a “test battery” approach may best determine a tumor’s individual growth capability. This strategy may more accurately predict a tumor’s responsiveness to surgical and medical treatments and various forms of adjuvant therapy.

Conventional histopathologic evaluation of bladder cancer, including determination of tumor grade and stage, is now inadequate to accurately predict the behavior of many bladder cancers, despite the significant progress made in the development and evaluation of tumor markers. Currently, firm recommendations cannot be made about most of the markers for the management of bladder cancer. This inability relates to the present maturity of methodologic principles seen in prognostic factor studies.

However, molecular techniques used in the study of tumor markers will continue to evolve at an ever-increasing pace. The need for well-designed, randomized, prospective clinical trials evaluating the strongest candidate markers remains. It will benefit all clinicians to strive to gain a basic understanding of tumor cell biology, ensuring that there is adequate knowledge of the importance of these trials. This knowledge will improve patient accrual, condense the time necessary to obtain definitive results, and eventually allow for improved patient care and outcomes.

**Conclusions**
With a better understanding of the cell cycle, tumor-suppressor genes, and cell-to-extracellular matrix interactions, progress is being made in the identification and characterization of other potential prognostic markers for patients with transitional cell carcinoma of the bladder. The ultimate goal is to develop reliable prognostic markers that will accurately predict not only the course of an individual bladder tumor but also the response of that tumor to therapy. In the future, this information may be employed to dictate more aggressive treatment regimens for patients whose tumors are likely to progress and less aggressive treatment regimens for those unlikely to progress. Altogether new treatments also may be forthcoming for the prevention and/or stabilization of early molecular events leading to the development of bladder cancer.

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