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INVITED EDITORIAL

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Molecular pathways in bladder cancer

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Abstract The aim of this review is to provide a contemporary outline of our current understanding of the molecular and genetic events associated with tumorigenesis and the progression of bladder cancer. A comprehensive review of the literature was performed on the molecular alterations associated with transitional cell carcinoma (TCC) of the bladder. Intense research efforts are being made to better identify and characterize various bladder cancers and their true biologic potential. The need to predict which superficial tumors will recur or progress, and which invasive tumors will metastasize has led to a much better understanding of the molecular pathways associated with bladder cancer. The molecular changes that occur in TCC of the bladder are numerous and can be categorized into: (1) chromosomal alterations leading to carcinogenesis, (2) loss of cell cycle regulation accounting for cellular proliferation, and (3) metastasis, guided by events such as angiogenesis. It is becoming apparent that the accumulation of genetic and molecular changes ultimately determines a tumors phenotype and subsequent clinical behavior. At the present time, conventional histopathologic evaluation of bladder cancer (tumor grade and stage) is inadequate to accurately predict the behavior of most bladder tumors. While new laboratory techniques have allowed us to better understand how bladder cancer develops and ultimately progresses, few of these techniques are currently available for use in the clinical setting. The ultimate goal is to develop reliable prognostic markers which will accurately predict not only the expected clinical course of an individual bladder tumor but also the response of that tumor to currently available therapies. More importantly, this information may be employed in the future to dictate altogether new treatments for the prevention and/or stabilization of the early molecular events that lead to the development of bladder cancer.

Keywords Molecular pathway · Genetic events · Bladder cancer · Tumorigenesis · Transitional cell carcinoma

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Transitional cell carcinoma (TCC) of the bladder is the second most common malignancy of the genitourinary tract, and the second most common cause of death of all genitourinary tumors. In 2003, there were 42,200 new cases of the disease diagnosed, with 8,600 patient deaths [1]. Approximately 80% of patients with primary bladder cancer present which low grade tumors confined to the superficial mucosa. The risk of recurrence in these patients with superficial bladder tumors can be as high as 70%, with the majority of cancers amenable to initial transurethral resection and the selected administration of intravesical immuno- or chemotherapy [2]. Unfortunately, as many as 30% of these recurrent tumors may demonstrate tumor progression to a higher grade and/or stage of disease. Furthermore, 15–30% of all patients with bladder cancer initially present with muscle invasive tumors, 50% of these patients who are treated locally for their invasive tumors will relapse with metastatic disease within 2 years of treatment [3]. These data underscore the heterogeneous nature and malignant potential of TCC of the bladder.

The optimal management of invasive bladder cancer requires the detection and accurate assessment of the tumor's biologic potential. Currently, histologic evaluation, including determination of tumor grade and stage, is the primary prognostic variable which dictates treatment strategies for patients with bladder cancer. Although these two conventional histopathologic variables

provide a certain degree of stratification of a tumor's biologic potential, there remains a significant degree of tumor heterogeneity even within various prognostic subgroups. This makes the accurate and reliable prediction of the tumor's aggressiveness difficult. The ability to precisely predict an individual tumor's true biologic potential would in turn facilitate treatment selection decisions for patients who may benefit from adjuvant therapy, and identify patients who may require less aggressive treatment strategies. Intense research efforts are ongoing to identify and better characterize bladder cancer and its varying biologic potential.

TCC of the bladder has generally been viewed as two different disease processes. Superficial bladder tumors are thought to be more of a locally proliferative, recurrent process, but can become invasive and even metastatic. The use of molecular markers may guide decision making processes in the treatment of superficial bladder cancer [4]. Superficial bladder tumors that maintain a malignant phenotype may be better treated with early, aggressive intravesical therapy, or even cystectomy. On the other hand, muscle invasive bladder cancer is notorious for its potential clinical virulence and is ideally treated aggressively [2]. Despite this aggressive form of therapy, there remains a significant incidence of recurrence and disease progression in patients, who may ultimately benefit from some adjuvant form of therapy.

The need to predict which superficial bladder tumors will recur or progress, and which invasive tumors will metastasize, has led to the ongoing attempt to understand 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 analyses of human cells and tissues at the DNA, RNA and protein levels. The molecular and genetic changes in TCC 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—due to 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 the loss of cell adhesion. We believe it is the accumulation of these successive genetic alterations, rather than a single genetic event in time, that determines a tumor's phenotype and, subsequently, the patients clinical outcome. In this review, we will summarize the recent literature on the molecular and genetic changes in bladder cancer and comment on potentially improved diagnostic abilities and treatment regimens that are becoming available as a result of our improved understanding of these molecular pathways.

Initiation of carcinogenesis—oncogenes and chromosomal alterations

Bladder cancer is an excellent model for the study of molecular changes at the DNA level, due 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 (LOH) [5]. DNA alterations can result from any number of genetic insults such as mutations (point and insertional/deletional), translocation, and loss of alleles. Each insult may effect the translated protein product. The large fund of molecular knowledge on carcinogenesis, developed in recent years, has provided some evidence of the different genetic pathways for bladder cancer.

Earlier work in the field of molecular oncology focused on oncogenes. Oncogenes 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 an 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, the 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 c-erbB2.

c-H-ras

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 codon 12 and 61 of the H-ras gene occur in up to 20% of bladder cancers [6, 7, 8]. One study employing polymerase chain reaction amplification followed by oligonucleotide specific hybridization reported that 36% of bladder tumors had the same mutation at codon 12 of the H-ras gene [9]. In general, the activation of H-ras occurs by a single point mutation (G to A) in condon 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

The myc gene family is an important regulator of cellular proliferation and encodes for nuclear phosphoproteins containing DNA-binding activity [11]. The c-myc oncogene is overexpressed in several human tumors including bladder cancer [12, 13]. Deregulation of the myc gene family occurs with chromosomal translocation and gene amplification [14], and studies have demonstrated that

myc overexpression promotes cellular proliferation [10]. Although the genetic mechanism causing overexpression of the c-myc gene in bladder cancer is unknown, overexpression of c-myc has been shown to be associated with high grade bladder cancer. Kotake and associates demonstrated that expression of the c-myc gene product correlates with the nuclear grade of bladder cancer [13]. In a conflicting study, Lipponen found no independent prognostic value for myc proteins with respect to the prognosis of patients with TCC of the bladder [15]. Currently, the prognostic significance of c-myc gene expression is unknown and further evaluation will be required to determine its prognostic role.

c-erB-2

The proto-oncogene c-erbB-2 (also known as HER-2/neu) has been extensively studied and implicated in a number of tumors including breast, prostate and bladder cancer [16]. The c-erbB-2 oncogene encodes a transmembrane glycoprotein, similar to the epidermal growth factor receptor, having tyrosine kinase activity [17] and the ability to stimulate cellular growth [18]. Initial studies of c-erbB-2 were performed in breast carcinoma, and demonstrated a significant relationship between gene expression, tumor progression and overall survival [19]. Subsequently, several studies have reported that c-erbB-2 expression in patients with bladder cancer is associated with higher stage tumors [20, 21, 22], increased tumor progression [16], increased incidence of metastasis [22], and decreased overall survival [20]. Although these studies suggest a prognostic value of c-erbB-2 expression in human bladder cancer, other studies have reported conflicting results, concluding that the evaluation of c-erbB-2 provides no additional prognostic value over previously established predictors (grade and stage) for TCC of the bladder [23, 24]. In view of these discrepant results, further evaluation will be required to accurately determine the prognostic value of c-erbB-2 in bladder cancer.

Tumor suppressor genes

More recent work in the field of chromosomal alterations has focused on identifying specific loci on chromosomes which may contain altered genes. Many of these genes have been identified as tumor suppressor genes (TSG) that, when inactivated, result in the initiation and/or progression of the malignant phenotype. Recently, with the advent of such techniques as LOH analysis and comparative genomic hybridization (CGH), a significant increase in genome scanning has occurred, with the identification of many new chromosomal alterations in TCC. LOH analysis uses known polymorphic markers to identify large deletions and/or alterations of both alleles of a chromosome. The most common scenario would be one large deletion of an entire chromosome due to natural genetic recombination, followed by a small alteration in the retained allele, involving an insertional, deletional, or point mutation, usually at a specific locus containing a tumor suppressor gene. CGH utilizes genomic DNA from tumor and normal cells that is differentially labeled by fluorescence. The two DNA extracts are then hybridized on to a platform of 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 to the normal hybridized DNA.

Using these molecular techniques, an extensive search in recent years has led to finding several key TSGs on different chromosomes. Deletions on the short arm of chromosomes 3 (3p) [25] and 8 (8p) have been found with high grade, muscle-invasive bladder cancer. In fact, the 8p deletion has been noted in $>50\%$ of muscle-invasive TCC [26, 27], (a rate similar to that of p53 mutations), while no deletions were noted in superficial TCC. LOH on the short arm of chromosome 8 (8p) has also been associated with high grades and stages of TCC, but the relevant gene(s) have not yet been identified. However, the most notable chromosomal deletions in bladder cancer have been found on chromosomes 9, 13, 17. This has led to the identification of the retinoblastoma (Rb) TSG on chromosome 13, the p53 TSG on chromosome 17, and promising new TSG on chromosome 9 at the p21 locus.

Deletions on chromosome 9 are the most common chromosomal abnormalities in TCC, and are found in $>50\%$ of all grades and stages of TCC. However, it is becoming clear that most muscle invasive bladder tumors have other chromosomal alterations as well. On the other hand, most Ta and T1 tumors show few genetic alterations other than on chromosome 9 [28, 29, 30]. This has led to the hypothesis that inactivation of genes on chromosome 9 may represent an early event in the development of bladder cancer.

The majority of deletions on chromosome 9 have been found on the short arm (9p). Specifically, a complex genomic region at 9p21 (INK4A/ARF and INK4B) exists, encoding three distinct proteins—p16, p14ARF, and p15—all of which act as negative cell cycle regulators and are considered potential TSGs [31]. Extensive screening of the retained allele in bladder tumors with 9p LOH have not revealed frequent mutations. Instead, deletion of both alleles (homozygous deletion) appears to be the common mechanism of inactivating the entire locus in TCC [32, 33, 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 shown growth arrest following the introduction of p16 into cell lines with deleted INK4A [35]. Identification of the p14ARF, p15, and p16 proteins, for use as markers of malignant potential, are currently under investigation, and will be discussed further in the section on cell cycle regulation.

Recent studies involving large LOH analyses have also identified possible loci for TSG on 9q, at 9913–31, 9932–33 and 9934 [75, 76, 80]. Candidate genes have been identified at 9932–33 (deleted in bladder cancer candidate region 1-DBCCR1) and 9934, the same region as the tuberous sclerosis gene. The DBCCR1 gene shows no significant homology to other known genes, and is expressed in the urothelium of adult bladder and ureter. However, in one study about half of the bladder tumor cell lines studied showed no expression of DBCCR1, perhaps indicating gene silencing secondary to DNA methylation in the promoter region [36]. Most investigators have found no relationship between 99 loss and tumor invasiveness, possibly supporting its role in the initiation of bladder cancer, rather than in progression [37].

Two other very important chromosomal alterations that affect known TSGs involve 17p13, the site of the p53 gene, and 13914, the site of the Rb gene. The importance of p53 in bladder TCC was suggested by the high frequency of LOH of chromosome 17p in high grade TCC [38]. Others have confirmed that p53 follows classic tumor suppressor theory, with LOH at one allele (17p) and a mutation of the remaining TP53 allele. This pattern has subsequently been identified in a large number of muscle-invasive bladder tumors, with a lower frequency in superficial tumors [39, 40]. These genetic defects have been demonstrated to correspond with protein expression of the mutated p53 gene product [41, 42]. However, p53 evaluation remains a good example of the difficulty of making the transition from genetic studies to translational research techniques such as immunohistochemistry (IHC). Nearly 25% of tumors that are p53-altered (positive) by IHC show no detectable mutations by standard gene sequencing analyses, while about 10% of wild-type tumors (p53 negative) harbor readily identifiable mutations [42, 43]. Still, p53 remains the best characterized TSG to date and is clearly implicated in disease progression in several solid tumors, including bladder cancer.

The Rb gene was the first TSG isolated. It codes for a nuclear protein mapping to 13914 [44]. Like p53, Rb is thought to play an important role in bladder cancer progression. Rb gene mutations are noted in 25–30% of bladder tumors [45, 46], and LOH at the Rb locus (13q) is strongly associated with the absence of Rb protein expression by IHC [47]. However, unlike p53, missense mutations are rare in RB and no mutational hotspots have been identified. In fact, mutations have been found distributed throughout 24 of the 27 exons in hereditary retinoblastoma. Therefore, detailed analyses of specific mutations within the Rb gene are unlikely to be used in clinical decision making as the large size of the gene does not lend itself to standard DNA sequencing or single strand conformational polymorphism analysis [48].

In a recent study, 12 of 19 bladder tumor cell lines had identifiable mutations in exons 5–11, considered the central domain, of TP53, with a concomitant loss of Rb protein expression. The other seven bladder cancer cell lines studied showed wild-type p53 (normal) or mutations only in exons 1–4, but all seven cell lines had concomitant alterations at the 9p21 (INK4A/ARF and INK4B) gene locus [49]. This study provided the first evidence for 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 et al. [50].

We have known for years that superficial and muscle-invasive TCC of the bladder are quite different histologically and that they behave altogether differently in the clinical realm. On the basis of the findings described above, of frequent and consistent genetic alterations in the two primary forms of bladder cancer, we are now able to come up with a fairly detailed model of these "multiple genetic pathways of bladder carcinogenesis" (Fig. 1). The key studies to date indicate that chromosomal instability leading to alterations in cell cycle regulation are integral events in determining the biologic behavior of bladder tumors. Although our understanding of the actual molecular events leading to the initiation of bladder cancer is growing rapidly, none of the techniques currently available to identify specific allelic instability or LOH in individual bladder tumors are yet applicable for clinical use. The advent of high-throughout molecular technology, however, may allow for clinical testing of specific chromosomal alterations in individual patients in the near future.

The cell cycle regulatory pathway

Normal cellular proliferation occurs by an orderly progression through the cell cycle which is regulated by cell cycle associated protein complexes composed of cyclins and cyclin-dependent kinases [51]. Several TSGs acting

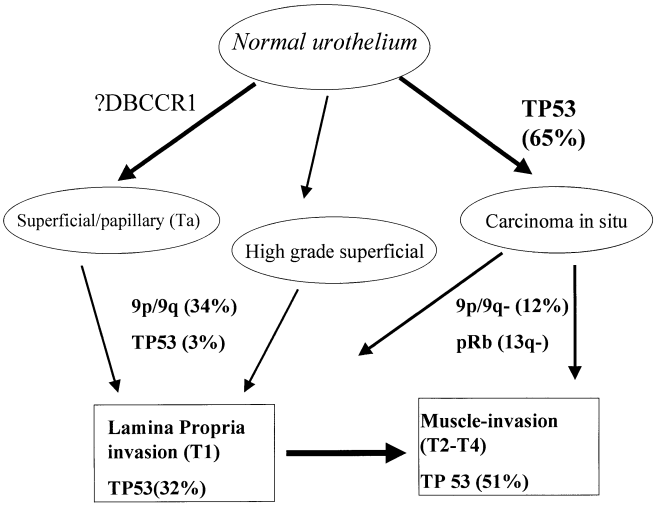

Fig. 1 Molecular model of bladder cancer and its progression showing key molecular alterations that have been described. Values in parentheses for chromosome 9 and TP53 mutation frequencies are from [50]. Adapted from [31]

at the G0/G1 checkpoint of the cell cycle are now recognized, and their protein products—p53, pRb, p16, and p14—are vital for preventing cell cycle progression in bladder tumors. Inactivation of one or more TSGs 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: p53 is an exception as the 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: (1) primary inherited alteration of one copy, followed by a second "hit" occurring somatically (due to environmental mutagen exposure or dysfunction of DNA replication/repair), or (2) an entirely somatic event(s) in which two independent "hits" occur in both copies of a gene [48].

While most of the initial studies on cell cycle regulation in bladder cancer focused on the role of individual TSG and their protein products, more recent investigative efforts have identified multiple pathways within the cell cycle. These pathways involve the interaction of multiple TSG, and it is this interaction that is most likely responsible for bladder cancer progression.

Retinoblastoma TSG

The Rb gene is located on chromosome 13914 and encodes for a 110 kDa nuclear phosphoprotein [52]. Although initially discovered to be mutated in patients with inherited retinoblastoma, altered Rb gene expression has been reported in various human tumors including TCC of the bladder [45, 46, 53]. In its physiologic active hypophosphorylated form, pRb acts by inhibiting cell cycle progression at the G1-S checkpoint. However, pRb interacts with multiple cell cycle regulatory proteins, including: (1) cyclins, which catalyze the inactivation of pRb via phosphorylation, (2) cdk inhibitors, including p21, p16, and p27, which activate cdk/cyclin complexes, thus inhibiting pRb phosphorylation, and (3) the E2F family of transcription factors, which are responsible for transactivating genes necessary for entry into the S (synthesis) phase of the cell cycle [54]. Any alteration in those 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 [47, 55]. The results of these studies suggest that the loss of pRb expression may be an important prognostic factor in TCC of the bladder. Cordon-Cardo and associates reported that patients with muscle invasive bladder tumors who had lost Rb immunoexpression had a significantly shorter 5-year survival than those patients which normal Rb protein expression [46]. Similarly, Logothetis and associates studied 43 patients with invasive bladder cancer and demonstrated that Rb alterations were more common in advanced tumors, and that those patients who had lost pRb expression had a shorter overall survival compared to those who had maintained Rb expression [45]. Based on the aforementioned data, it appears that pRb expression is an important prognostic factor in patients with invasive bladder cancer.

p53 TSG

Mutations in the p53 gene are the most common genetic defect in human tumors [56]. The p53 gene is located on chromosome 17p13 and encodes for a 53-kDa protein. The p53 gene is known to play a vital role in the regulation of the cell cycle [57]. 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 the nuclear accumulation of p53 protein, as determined by immunohistochemical staining, correlates with gene mutations detected by DNA sequence analysis [42, 58, 59].

p53 alteration, as determined by immunohistochemical techniques, is an important prognostic indicator for bladder cancer progression [41, 60, 61]. Increased p53 immunoreactivity has been found in higher grade and stage bladder cancers and is associated with disease progression and decreased 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 [60]. Patients with an increased p53 expression (altered p53) were found to have a significantly increased risk of disease recurrence and a significantly decreased overall survival when compared to those patients without altered p53. This association was strongest in patients with organ confined bladder tumors (P1, P2, P3a). Furthermore, nuclear accumulation of p53 was found to be the only independent predictor of disease progression in a multivariate analysis of p53 status, histologic grade and pathologic stage.

Evidence is now accumulating 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 alterations in exons 5–8, the central core domain of p53 [3]. However, the frequency of mutations outside this region (exons 2–4 and 9–11), although lower, is highest in bladder cancer compared to other cancers [62]. Markl and Jones have shown, in bladder cancer cell lines, that mutations in

exon 4 of p53 were always associated with loss of p14/p16, while mutations in exons 5–11 were always paired with loss pRB [49].

Combination of Rb and p53 TSGs

Two independent studies have evaluated the prognostic significance of combining the Rb and p53 status of bladder cancers as determined by immunohistochemical techniques [63, 64]. Preliminary data from these studies support the concept that bladder tumors with alterations in both p53 and Rb have a poorer prognosis and decreased overall survival when compared to tumors with wild-type p53 and wild-type Rb. Tumors with an alteration of only one of these genes (as determined by immunohistochemistry) 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 which bladder cancer.

p21 TSG

Although p53 nuclear accumulation, as detected by immunohistochemical methods, is a significant predictor of bladder cancer progression, not all p53-altered bladder tumors recur or progress [61, 60]. One of the primary functions of p53 is as a cell cycle regulatory protein [59]. p53 mediates its effects on the cell cycle, in part, through the regulation of $p21^{WAF1/CIP1}$ expression [51]. Alterations in p53 can result in loss of p21 expression, which leads to unregulated cell growth. This is thought to be one of the mechanisms through which p53 alterations may influence tumor progression. However, it has recently been demonstrated that p21 expression may also be mediated through p53-independent pathways [65, 66]. This important finding suggests that despite the presence of a p53 alteration, p21 expression (and therefore cell cycle control) can be maintained.

We have evaluated bladder tumors from 101 patients who underwent radical cystectomy for invasive bladder cancer for p21 expression using immunohistochemical techniques [66]. All patients had been previously determined to have p53 altered tumors [60]. We found that immunohistochemical detection of p21 protein in the nuclei of bladder cancers which show p53 alterations (p53-altered) provides important additional prognostic information for patients with bladder cancer. Patients with p53-altered TCC of the bladder that were p21-negative demonstrated a significantly increased probability of recurrence and a significantly decreased probability of overall survival when compared to patients with p53-altered tumors that maintained expression of p21 (p21-positive). The association between p21 status and prognosis in p53-altered bladder tumors was independent of tumor grade, pathological stage and lymph node status. Loss of p21 expression was strongly associated with an increased probability of recurrence and decreased probability of 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 exist, and that cell cycle control may be maintained through these pathways. Those patients with p53 altered tumors that lose p21 expression appear to have a poor prognosis and may best be managed with aggressive forms of therapy.

Deciphering cell cycle regulation in TCC

The interaction of p53 and p21 in cell cycle regulation, and the data looking at the cooperative effects of p53 and Rb, provide good examples of the increasing evidence that mutation in a single TSG is unlikely to be the only factor resulting in carcinogenesis. We now understand that there are several pathways within the cell cycle, each playing a role in cell cycle regulation. The alteration of one or more of these pathways is likely responsible for bladder cancer progression.

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

p16 (also known as INK4A, MTS1, CDKN2A) is a well-characterized CDK inhibitor [67] which functions upstream of pRb to block cyclin-D directed phosphorylation of Rb, thus inducing G1 arrest. p16 mutations and homozygous deletions are common in bladder cancer cell lines and in squamous cell carcinoma and bilharziasis associated bladder cancers [54]. Furthermore, p16 is thought to be susceptible to transcriptional silencing by promoter methylation [68]. 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, also known as ARF (alternative reading frame), and as p19 in the mouse. p14 acts upstream of p53 to stimulate p21 expression, and 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 [69]. However, p14 is a very different TSG than p16 in that it is expressed ubiquitously, whereas p16 expression is more restricted; p14 does not bind to CDKs or function as a CDK inhibitor [70], and it can cause cell cycle arrest at any point in the cycle through its effect on p21 [48].

While the majority of research on the 9p21 locus has been performed in vitro using cell lines and animal models, Orlow et al. 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 a lower recurrence-free survival. Furthermore, deletions that affected both p16 and p14 (thus deregulating both the p53 and pRb pathways), correlated with larger and higher grade tumors [71].

As previously mentioned, p14 regulation of the cell cycle can occur through physical interaction with MDM2 [72]. In normal cells, MDM2 regulates p53 function by marking p53 for degradation via ubiquitin conjugation and inactivating p53 by binding to its transactivation domain [73]. p14 binding of MDM2 appears to counteract the effects of MDM2 by protecting p53 from degradation [73]. Nevertheless, the role of MDM2 in regulating p53 protein levels in TCC of the bladder remains unclear. The frequency of MDM2 gene amplification across all malignancies is thought to be uncommon (~7%), with the highest frequency in soft tissue tumors (20%). MDM2 amplifications and p53 mutations usually do not occur within the same tumor sample, indicating that carcinogenesis can result from MDM2 amplification alone. However, MDM2 gene amplification is infrequent in bladder cancer, with one study showing only one of 87 cases of MDM2 amplification, despite elevated MDM2 protein levels in 26 of the 87 cases [74].

Thus, the role of TSGs in cell cycle regulation is a complex one, with the 9p21 gene locus lying at the center of the two major tumor suppressor pathways identified in bladder cancer—p53 and Rb. The two pathways and the various TSG interactions described above are summarized in Fig. 2.

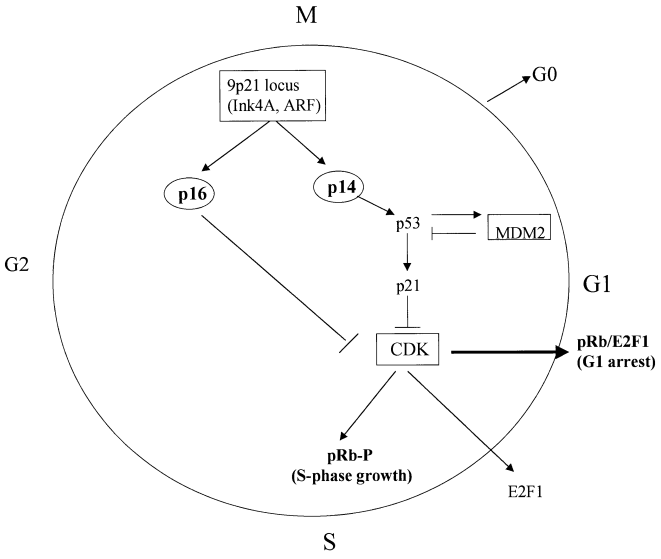

Fig. 2 The INK4A/ARF locus and cell-cycle regulation. The 9p21 locus yields two unique proteins, p14 and p16, which act along separate pathways of cell cycle control. p14 modulates expression of p53 by directly stimulating expression or indirectly downregulating expression via MDM2. p53 then upregulates expression of p21, inhibiting the cyclin-dependent kinase (CDK)-cyclin complex from phosphorylating pRB and leading to Gl-phase arrest. The p16 pathway leads to direct inhibition of the CDK-cyclin complex, yielding cell cycle arrest through inhibition of pRB phosphorylation

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 cells, (2) migration and invasion of the activated endothelial cells following proteolytic degradation of the surrounding extracellular matrix, and (3) maturation of the endothelial cells to coalesce and form water tight tubules that establish new blood flow [75]. 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 (neovascularization) is a critical adaptation characteristic of all solid tumors. Without angiogenesis tumor growth is inhibited at a diameter of 2–3 mm, the natural limit for diffusion of essential nutrients and oxygen [76]. Under most homeostatic conditions, new blood vessel formation is infrequent and is controlled by an abundance of inhibitory signals directed at the endothelium, setting the balance in favor of vascular quiescence. It is thought that the angiogenic phenotype for any given tumor is determined by the overall balance between stimulatory and inhibitory inputs to the endothelial cells. 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", whereby new vessel growth is stimulated, thus providing the necessary nutrients for continued tumor growth and eventual metastasis [77]. Current research has identified several mechanisms by which this angiogenic switch can occur, including overexpression of inducers and/or loss of endogenous inhibitor production [78]. 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 [79, 80]. 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 CD 34, which 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 [81], breast cancer [82], and prostate cancer [83]. In general, increased microvessel density counts have been associated with tumor progression and decreased survival [83, 84]. The relationship between microvessel density count and tumor progression has also been examined in patients with bladder cancer [85, 86]. Dickinson et al. evaluated a series of 45 patients which invasive bladder tumors with a median follow-up of 37 months, and found microvessel density count to be an independent prognostic indicator of disease progression. Patients which an elevated microvessel density count demonstrated a 2.5-fold greater risk of dying [87].

Our group recently evaluated the relationship between tumor angiogenesis and tumor progression in 164 patients with invasive bladder cancer [85]. In this study, microvessel density was significantly associated which 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 when compared to patients with low microvessel density counts. Furthermore, microvessel density count was found 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

The difficulty in evaluating the angiogenic potential of any given tumor results from the abundance of pro-angiogenic factors that are produced by tumor cells or released by the surrounding extracellular matrix (ECM) [88] (Table 1). Prevailing evidence for the balance hypothesis proposed by Folkman suggests that an angiogenesis suppressor gene(s) encodes or controls the 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, resulting in a balance in favor of the angiogenic inducers, with subsequent endothelial cell proliferation and migration (thus the term "angiogenic switch") [77].

The angiogenic properties of urine from patients with TCC were first noted by Chodak and associates who documented a stimulatory effect on the migration of endothelial cells exposed to the urine [89]. Basic fibroblast growth factor (bFGF), a potent pro-angiogenic factor, is excreted at higher levels in the urine of bladder cancer patients compared to patients without evidence of disease [90, 91]. Urinary bFGF has also been correlated with the pathologic stage of the primary tumor in patients with muscle-invasive TCC [92].

Recently, elevated levels of another important angiogenic inducer—vascular endothelial growth factor (VEGF)—have been found in the urine of bladder cancer patients. Crew and colleagues evaluated 98 patients with bladder cancer and found 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 [93]. We recently found elevated levels of VEGF in the urine of bladder cancer patients with high grade and/or muscle-invasive TCC, as compared to patients with prostate cancer and patients without evidence of malignancy. In 92 patients undergoing radical cystectomy for muscle-invasive or high grade superficial TCC of the bladder, higher VEGF levels in urine obtained preoperatively were associated which significantly decreased 3 year survival [94].

While numerous pro-angiogenic factors have been identified in bladder cancer cell lines and tissue, Campbell and colleagues have shown that VEGF and bFGF appear to be two primary inducers of angiogenesis in bladder cancer cell lines [88]. Neutralizing antibodies to VEGF, and to a lesser extent bFGF, significantly reduced the angiogenic activity of bladder cancer cell lines, whereas neutralizing antibodies to aFGF, scatter factor, TGF alpha and beta, and thymidine phosphorylase did not.

O'Brien and colleagues found that tissue levels of VEGF taken from human bladder cancer tumors correlated with the stage progression in superficial TCC. They found a fourfold increase in VEGF mRNA levels in Ta tumors compared to normal urothelium, and a tenfold increase in T1 tumors. Interestingly, T1 tumors also had an increased expression of VEGF mRNA when compared to invasive (T2–T4) tumors [95]. Other researchers have found a similar expression of VEGF in normal urothelial tissue and bladder cancer. Campbell et al. found relatively constant levels of VEGF immunostaining across normal urothelium, superficial and muscle-invasive bladder cancer [88]. Sato et al. found that the VEGF transcript was present in both normal urothelium and bladder cancer tissue. However, muscle-invasive tumors expressed significantly higher levels of VEGF by Northern blot analysis [96].

In most cases, angiogenic factors appear to be produced directly by the bladder cancer cells. Interestingly, the immunostaining pattern for bFGF appears to be unique in that bFGF localizes primarily to the basement membrane rather than tumor cells. O'Brien and colleagues hypothesized that tumor-induced degradation of the basement membrane could release bFGF, accounting

Table 1 Proangiogenic factors identified in bladder cancer

Proangiogenic factors	Acidic fibroblast growth factor [119, 120]
	Basic fibroblast growth factor [90, 92]
	Vascular endothelial growth factor [88, 95, 121]
	Thymidine phosphorylase [95, 122]
	Scatter factor [123]
	TGF beta 1 and 2 [124]
	Interleukin 8 [125]
	Matrix-degrading enzymes [110, 126]

for the increased levels found in the serum and urine of bladder cancer patients [97].

Angiogenesis inhibitors

While much of the research on bladder cancer angiogenesis to date has focused on inducers, we know, based on the balance hypothesis, that this represents only part of the puzzle of predicting a tumor's metastatic potential. Many inhibitors of angiogenesis exist, including thalidomide [98], interleukin-12 [99], angiostatin [100], and thrombospondin-1 [101, 102]. While angiostatin has been shown to inhibit the growth of Lewis lung carcinoma [100], human breast cancer [103], human colon cancer [103], and human prostate cancer [104] in animal models, only thrombospondin-1 has been examined in human bladder cancer.

Thrombospondin-1 (TSP) is an extracellular matrix glycoprotein that has been shown to be a potent inhibitor of angiogenesis, both in vitro and in vivo [105, 106]. Campbell and colleagues showed 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 to TSP-1 [88]. Our group reported that TSP expression can be determined using antigen retrieval immunohistochemistry in routinely processed formalin-fixed, paraffin-embedded tissue [107]. Employing this technique, we evaluated 163 patients which invasive bladder cancer for TSP expression. Patients with low TSP expression exhibited higher recurrence rates and decreased overall survival when compared to patients which moderate or high TSP expression. This association was strongest in those 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 addition, in this same cohort of patients, we found that tumors with a low TSP expression were significantly more likely to demonstrate high microvessel density counts [108].

The extracellular matrix and angiogenesis

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 ECM serves to maintain endothelial cell function, and provides a scaffolding through which the endothelium may attach and migrate during capillary formation. Joseph and colleagues have shown that bladder cancer cells can induce the production of scatter factor (a known angiogenesis inducer) by the underlying stromal cells [109]. Matrix metalloproteinases (MMP) are also thought to play an important role in the degradation of the ECM. MMP-2 and MMP-9 are elevated in the serum and urine of patients with muscle-invasive bladder cancer, and are correlated with poorer disease free survival [110]. Furthermore, MMP-9 has an increased expression in TCC when compared to normal urothelium, and also correlated with increasing tumor stage [111].

Anti-angiogenic therapy

As a result of the improved understanding of tumor angiogenesis, clinical trials have now begun for some solid tumors in an attempt to develop effective anti-angiogenic therapies. Targeting the activated vessels associated with neovascularization of solid tumors provides several advantages over conventional forms of treatment. First, all blood vessels are readily accessible via the circulation, and are required by all tumors for growth and metastasis, yet are not necessary for normal physiologic function except for wound healing and female fertility [112]. Secondly, the endothelium targeted by an anti-angiogenic approach is non-neoplastic and maintains its full compliment of regulatory mechanisms. Endothelial cells are unlikely to undergo the changes found in solid tumors that allow for the development of drug resistance [113]. Furthermore, there is a paucity of side effects associated with anti-angiogenic therapy—no bone marrow suppression or gastrointestinal mucosal alteration [114] making 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 anti-tumor activity in animals [115]. All hereditary and most sporadic (clear cell) renal cell carcinomas (RCC) are associated with a defect in the VHL tumor suppressor gene located on chromosome 3p. A function of the VHL gene regulates the expression of proteins, including suppressing the expression of VEGF. Following a mutation in the gene or a partial deletion of chromosome 3, VEGF overexpression occurs and is thought to contribute to the many vascular manifestations characteristic of VHL syndrome [116]. Anti-VEGF therapy is now under investigation as a novel therapy against metastatic renal cell carcinoma [117].

While no clinical trial utilizing anti-angiogenic agents in bladder cancer has yet been established, the potential benefits are obvious. Anti-angiogenic therapy could be used for chemoprevention in patients at high risk for recurrence or progression, possibly through intravesical administration. A second indication would be for adjuvant therapy in patients at high risk for progression (advanced tumor stage, regional nodal involvement, altered p53) following radical cystectomy. This is particularly appealing, as the tumor burden is relatively low, and experimental models suggest that maintaining micrometastases in a dormant state for prolonged periods of time may be a reasonable goal, especially for older patients. Younger patients would likely require long-term or

intermittent administration of anti-angiogenic agents to maintain a disease-free or disease-stable state [118].

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, tightly coordinated process not yet fully understood. While 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 only looking at the tip of the iceberg in understanding how complex cellular interactions lead to tumor progression and metastasis. While therapies directed at individual inducers hold promise, this complexity of the angiogenic process makes it unlikely that targeting a single inducer will be an adequate treatment. Nevertheless, improved understanding of the molecular pathways regulating angiogenesis will undoubtedly improve tumor prognostication and treatment options.

Future directions

For now, it is clear that conventional histopathologic evaluation of bladder cancer, including determination of tumor grade and stage, is inadequate to accurately predict the behavior of many bladder cancers. One goal of understanding the biology of urothelial cell transformation and disease progression is to provide diagnostic markers that will help predict a tumor's natural history as well as its response to treatment. As we begin to decipher the molecular events that determine malignant transformation, cellular proliferation, and ultimately metastasis, we have come to realize the incredible complexity of bladder cancers. As a result, it is becoming obvious that diagnostic and therapeutic capabilities cannot be based on the knowledge of a single gene or protein, but rather on a complete understanding of chromosomal alterations, cell cycle activity, and extracellular interactions. Still, we are beginning to witness the emergence of molecular markers that provide some information on an individual tumor's biologic potential. Initially, these markers will help the transition from molecular science to clinical application, guiding therapeutic treatment plans for individual patients. This will indeed herald a new era, integrating molecular biology with surgery, pathology, and medical oncology for the purpose of disease management. Ultimately, this integration will require well designed prospective clinical trials of tumor markers and therapeutic drugs, if medicine is to truly benefit from this ''molecular revolution''. Thus, it behooves all urologists, clinicians and scientists alike who are involved in bladder cancer management to gain an understanding of urothelial cell tumor biology, so that there is an adequate knowledge of the importance of these trials. This will improve patient accrual, decrease the time necessary to obtain definitive results, and eventually allow for improved patient care and outcomes.

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