Molecular markers in bladder cancer
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**Purpose of review**
Bladder cancer is a diverse disease whose molecular phenotypes are being elucidated. In this review, we summarize currently known molecular pathways and associated markers in bladder cancer.

**Recent findings**
Genetic and epigenetic aberrations have been closely associated with tumor pathogenesis and prognosis. Cell cycle markers have been most extensively studied. More recently, apoptotic and angiogenic pathways are being investigated. Studying the role of multiple concurrent molecular alterations improves the prognostic ability of these markers. The use of tissue microarrays and high-throughput molecular profiling is accelerating the discovery of new markers.

**Summary**
Molecular biology is paramount to our understanding of bladder cancer pathogenesis. The search for new markers, and elucidating cross-talk between markers in different pathways, is warranted. Molecular markers have the potential benefit of improving detection, prognosis and treatment of bladder cancer. In addition, understanding the molecular profile of the individual patient could usher us into a new era of improving prediction of the natural history of the disease and providing a more personalized and tailored treatment. Prospective trials are still needed, however, to objectively establish the true benefit of these markers in prognostic and therapeutic arenas.

**Keywords**
bladder cancer, marker, molecular biology, prediction

**Introduction**
Intensive research over the last decade including the descriptive and mechanistic molecular studies of bladder cancer has provided great insight into the biology of this cancer and is beginning to shape clinical practice. Technologies such as high-throughput transcript profiling, microarrays, and proteomics, have facilitated the comprehensive identification and understanding of molecular pathways and targets that are active in bladder cancer. Molecular profiling of bladder cancer is starting to offer alternative means to comprehend tumor behavior and thereby improve outcomes for patients. In addition, for new therapeutic approaches to flourish there is a need for new markers to serve as prognosticators or rational targets for drug development.

It is now clear that bladder cancer develops along complex molecular pathways. Spruck \textit{et al.} \cite{1} first proposed a framework to address a dynamic model of molecular events involved in the series of states from diagnosis to death. Each of the steps associated with malignancy, including tumorigenicity and oncogenicity (ability of a cancer to proliferate continuously in absence of the persistent stimulant), tumor progression (evolution of already tumorigenic cells toward increasingly autonomous states), and metastatic propensity are rife with multiple genetic and epigenetic events indicating that few tumors are genetically or phenotypically identical. This review briefly explores recent advances regarding the biology and clinical utility of potentially useful prognostic markers.

**Genetic alterations**
Multiple genetic and epigenetic alterations have been described in bladder cancer. While alterations of chromosome 5 have been described in bladder cancer \cite{2,3}, recent studies narrow the abnormal area to the 5p13 region \cite{4,5,6}. This region contains \textit{DOC-2/DAB2} (\textit{differentially expressed in ovarian carcinoma-2/disabled-2}), a candidate tumor suppressor gene originally identified via a search for differentially downregulated transcripts in human ovarian carcinoma \cite{7,8}. The \textit{DOC-2/DAB2} gene has been cloned...
and localized to chromosome 5p13 [9]. Downregulation of DOC-2/DAB2 has been reported in bladder cancer cell lines [10], transgenic mouse models, and human specimens [11].

Another chromosomal alteration reported in bladder cancer is that of chromosome 8, which may involve loss of the p arm, or gain of the q arm. Loss of heterozygosity of 8p is associated with more aggressive tumors. The 8p21–22 locus contains several candidate genes: DBC2, LZTS1, and TRAIL-R2 [12,13]. A commonly gained region in 8q24 involves the c-myc proto-oncogene. Higher c-myc copy number was associated with advanced tumor stage and grade; however, c-myc copy number changes did not correlate with prognosis of bladder cancer patients [14].

Chromosome 9 abnormalities are typically present in the early stages of bladder tumorigenesis [1,15,16]. Alterations of chromosome 9 are thought to predispose cells to acquire more advanced chromosomal and genetic abnormalities, thus facilitating disease progression [17]. Potential chromosomal loci for tumor suppressor genes are at 9p21, which contains CDKN2A (p16/INK4A), at 9q12–31 which contains PTCH1 (Patched 1), at 9q32–33 which contains DBC1 (deleted in bladder cancer 1), and at 9q34 which contains TSCI [18].

The cell cycle
Mutations of cell cycle regulatory genes are the genetic alterations most commonly found in bladder cancer [19–22]. Various researchers have reported that alteration of p53, pRB, p21, and p27 expression have prognostic significance in patients treated with radical cystectomy for bladder cancer [23–31].

The p53 signaling pathway
The tumor suppressor p53 is known as the ‘guardian of the genome’ owing to its ability to integrate many signals that control life or death [32]. While most of the published material supports that p53 nuclear accumulation is predictive of outcome particularly for patients treated with radical cystectomy [30,31,33], there is evidence for and against almost every aspect of the role of p53. The fact underlying the discrepancies between studies is related to the choice of antibody, variability in the interpretation and stratification criteria, and inconsistency in specimen handling and technical procedures. Malats et al. [33] reviewed the published p53 studies and noted that altered p53 expression was an independent prognostic factor for tumor recurrence in nine of 34 reports, for progression in 12 of 24, and for survival in 10 of 35. Several recent small studies reported no association between p53 immunohistochemistry and outcome [34,35]. In a large tissue microarray study containing specimens from over 400 bladder cancer patients, however, the proportion of altered p53 gradually increased from normal urothelium to non-muscle-invasive bladder cancer to carcinoma in situ to muscle-invasive bladder cancer, and was highest in metastatic lymph node specimens [36–38]. In accordance with all large studies published to date [23–31], p53 expression was significantly associated with tumor stage, lymphovascular invasion, lymph node metastases, tumor grade, disease recurrence, and bladder cancer-specific death. Interestingly, the p53 phenotype was a stronger predictor of bladder cancer outcomes (disease recurrence and survival) in patients treated with radical cystectomy than p21, pRB, p27, p16, cyclin E1, or cyclin D1 [36–38].

The difficulty to accurately identify the life-threatening subgroup of patients with high-grade urothelial carcinoma involving the lamina propria (stage T1) represents a particular challenge for both physicians and patients. While some authors have shown that p53 immunohistochemistry may help stratify patients with T1 tumors into different risk groups with regards to clinical outcomes [39], others did not [40]. A preliminary report from the EPICURO confirmed that p53 overexpression correlated with higher grade and stage of the disease in 995 patients with non-muscle-invasive bladder cancer [41*]. The prognostic significance of p53, however, did not prevail in multivariate analysis. In a follow-up study, using both immunohistochemistry and gene sequencing of 119 tumors, the authors found that the p53 pathway is inactivated in most T1 grade 3 tumors, but failed to identify any prognostic value for p53 in these patients [41*]. Moonen et al. [42*] also reported no additional value for p53 mutation analysis over known prognostic factors using specimens from 105 patients with high-risk non-muscle invasive bladder cancer. Another group reported that p53 nuclear accumulation was not predictive of response to intravesical Bacillus Calmette–Guérin with or without interferon-α, cancer recurrence, progression, or cancer-specific survival [43*]. Finally, a recent prospective trial found no clinical utility for p53 expression in predicting survival in high-risk patients with T1 tumors, but this may have been affected by the low event rate related to treatment efficacy [44**].

PRB
Retinoblastoma has a pivotal role in stem cell maintenance, tissue regeneration, differentiation, and developmental programs. Retinoblastoma loss or inactivation is a major mechanism by which cancer cells attain a growth advantage during tumorigenesis. Recent studies, however, suggest that the predictive power of PRB may be inferior to other cell cycle regulators both in nonmuscle invasive and muscle-invasive bladder cancer [35–38].

P27
P27Kip1 is a member of the Cip/Kip family of Cdk inhibitors [45], which binds to Cdk2 (and to other Cdns) and
potently inhibits Cdk2 kinase activity. P27 overexpression in human cells has been shown to lead to cell-cycle arrest in the G1 phase. Two studies found limited predictive value for p27 in patients with nonmuscle invasive bladder cancer [37,46]. In patients with muscle-invasive disease treated with radical cystectomy, however, p27 was the second most powerful cell cycle regulator after p53 for prediction of bladder cancer recurrence and survival [36,38].

Cyclins
The deregulation of G1/S transition with the disappearance of restriction point is the hallmark of cancer, leading to continuous, uninhibited cell proliferation. Cyclins D and E are responsible for the initial and terminal phases of G1, respectively. In agreement with previous studies [47,48], normal bladder urothelium expresses cyclin E1 only in the umbrella cells, while cancer cells expressed cyclin E1 already in the earliest stages of the disease process [36]. Cyclin E1 expression was significantly decreased in patients with features of biologically and clinically aggressive bladder transitional cell carcinoma including advanced pathologic stage, lymphovascular invasion, metastases to regional lymph nodes, and bladder cancer-specific mortality in both transurethral resection of the bladder and radical cystectomy patients.

The same authors reported that cyclin D1 immunoreactivity was elevated in bladder cancer patients compared to controls but within bladder cancer patients it was not associated with clinical or pathologic characteristics [36]. While loss of cyclin D1 expression was associated with an increased probability of disease recurrence and bladder cancer-specific mortality in univariate analyses, this association was not significant when tested in a multivariate analysis that adjusted for the effects of standard pathologic features. These findings are consistent with previous reports showing that alteration in cyclin D1 is an early event in bladder tumorigenesis, but it does not add any prognostic significance.

Lopez-Beltran et al. [49] reported that cyclin D3 overexpression was associated with larger tumor size, tumor grade, and increased risk of disease progression (together with cyclin D1) in patients with Ta/T1 bladder cancer [49].

Apoptosis
Apoptosis, or programmed cell death, is a process that results from interplay of intrinsic and extrinsic signals that converge into a common downstream effector pathway [50]. Alterations in apoptosis pathways contribute to tumorigenesis and progression, as they allow cancerous cells to survive longer, resist normally harmful stresses, and become more invasive [51]. Bladder cancer has been shown to resist programmed cell death both with altered expression of proapoptotic and antiapoptotic proteins [30,50,52].

Fas (CD95)
Fas is an established death receptor. When activated by Fas ligand (FasL), Fas activates downstream signals that induce apoptosis [53]. Svatek et al. [54] reported that the soluble form of Fas was present in the cell lysate and supernatant of high-grade bladder cancer cell lines suggesting that it is likely to be produced and released by bladder cancer cells. In vivo, urinary soluble Fas was an independent predictor of bladder cancer presence and invasiveness in patients with a past history of nonmuscle-invasive disease. Urinary soluble Fas outperformed NMP22 for both bladder cancer detection and staging. These observations suggest that soluble Fas may play a role in the local immunosuppression in bladder cancer associated with tumor development, biologic aggressiveness, or progression.

Immunohistochemical analysis of the Fas system on 123 bladder cancer specimens and 30 benign bladders revealed that Fas was expressed in 90% of benign specimens compared to 58% of cancer specimens [55]. In addition, decreased Fas expression was associated with higher tumor stage, grade, and disease-specific mortality in univariate analysis [55].

Caspase-3
Caspase-3 is a downstream apoptosis effector molecule that causes cellular disassembly [56]. Giannopoulou et al. [57] studied caspase-3 expression in 53 patients with bladder cancer and did not find a correlation between caspase-3 expression and tumor grade or stage. Burton et al. [58] evaluated caspase-3 expression in 34 patients with carcinoma in situ, of which 41% developed invasive bladder cancer. They reported that activated caspase-3 overexpression was associated with higher rates of disease invasiveness. Conversely, a more recent study by Karam et al. [59] involving 226 consecutive patients treated with radical cystectomy specimens reported that 49% of the patients had loss of caspase-3 expression, which was associated with higher pathologic grade and stage, and presence of lymph node metastasis. Moreover, loss of caspase-3 was an independent predictor of bladder cancer-specific survival after radical cystectomy [59].

Bcl-2
Bcl-2 is an antiapoptotic protein present in intracellular membranes, and controls cytochrome c location, caspase status, and ion channels involved in apoptosis [60]. Overexpression of bcl-2 was found in 32% of radical cystectomy specimens, and correlated with higher pathologic stage, and disease recurrence and cancer-specific mortality rates [59]. In agreement with these findings, two other groups reported that overexpression of bcl-2 was associated with worse all-cause survival [61,62] and lower response rates to chemotherapy [62].
Survivin
Survivin is a newly discovered member of the inhibitor of apoptosis (IAP) family that inhibits apoptosis, at least partly, by blocking downstream caspase activity [63,64]. Survivin also controls mitotic progression and induces changes in gene expression that are associated with tumor cell invasiveness [65,66]. In bladder cancer, urinary levels of survivin gene activation, both at the protein and the mRNA level, have been shown to be associated with cancer presence, higher tumor grade, and advanced pathologic stage [67–69]. Survivin overexpression was present in 63% of bladder cancer specimens, and was associated with higher pathologic stage, lymphovascular invasion, lymph node metastasis, bladder cancer recurrence, and bladder cancer-specific survival in patients treated with radical cystectomy [59**]. In addition, the proportion of specimens with survivin overexpression increased gradually from nonmuscle invasive bladder cancer to advanced bladder cancer to metastatic lymph node tissue [70].

Angiogenesis
Angiogenesis, the process of new blood vessel formation, is a critical event in the initiation and progression of solid malignancies.

Microvessel density
Bochner et al. [71] evaluated 164 bladder cancer specimens and reported that patients with high microvessel density (>100 microvessels per high-power field) were at highest risk of recurrence (68% at 5 years) and bladder cancer-specific mortality (66% at 5 years) compared to patients with lower microvessel density. In addition, microvessel density was an independent prognostic factor when adjusted for the effect of tumor stage, grade, and lymph node status.

Thrombospondin-1
Thrombospondin-1 is a potent inhibitor of angiogenesis [72]. Thrombospondin-1 expression was also evaluated in the same patient population as microvessel density [71]. Its expression was inversely related to microvessel density. Moreover, thrombospondin-1 expression was an independent predictor of bladder cancer recurrence and all-cause survival [73].

Vascular endothelial growth factor
The vascular endothelial growth factor (VEGF) family is an important determinant of angiogenesis. In a study of 45 patients, VEGF-C expression was localized to the cytoplasm of bladder cancer cells, with minimal expression in normal bladder epithelium [74]. VEGF-C expression was significantly associated with tumor size, stage, and grade, lymphovascular invasion, and pelvic lymph node metastasis. In multivariate analysis, VEGF-C expression was an independent predictor of pelvic lymph node metastasis. Additionally, patients with high VEGF-C expression had a worse prognosis than those with low VEGF-C expression [74]. In a separate study of 126 patients who underwent transurethral resection of bladder cancer, higher expression of VEGF-A, VEGF-C, and VEGF-D were all three associated with increasing tumor stage and grade. As expected, VEGF-C expression was also associated with higher microvessel density.

Fibroblast growth factor receptor 3
Fibroblast growth factor receptor 3 (FGFR3) belongs to a tyrosine kinase receptor family that regulates diverse cellular processes such as growth, differentiation, and angiogenesis. Activating FGFR3 mutations are present in up to 60% of bladder cancer and are characteristic of papillary, nonmuscle invasive bladder cancer [75]. In general, FGFR3 mutation represents a subset of low grade/stage tumors that rarely progress and hence have a good prognosis [76,77]. Lindgren et al. [78*] used expression profiling, mutation analysis and loss of heterozygosity to molecularly characterize a cohort of 75 Ta and T1 bladder cancers. They confirmed that low grade/stage bladder cancer is characterized by FGFR3 receptor activity, either by mutation or by expression, high protein synthesis and low cell-cycle activity. Screening using microarray technology for FGFR3 and TP53 mutations by direct bidirectional sequencing and for genome-wide molecular changes of 35 Ta/low grade and 50 T1 or grade 3 tumors, Zhau et al. [79] found that FGFR3-mutated nonmuscle invasive tumors actually do progress and retain their mutation and chromosomal instability patterns during progression. Their results provide evidence that the decreasing frequency of FGFR3 mutations in later stages of tumor development is caused by the emergence of tumors following a different molecular pathway with no FGFR3 mutations. The tumors of this latter pathway were associated with carcinoma in situ. They further showed that the tumors within each pathway are molecularly related in terms of chromosomal instability and expression patterns. Finally, Lamy et al. [34] using systematic screening for FGFR3 and TP53 mutations reported that activation of FGFR3 and inactivation of TP53 are involved in the oncogenesis of nonmuscle invasive and invasive bladder tumors, respectively.

Combination of molecular markers
Given the complexity of the molecular abnormalities associated with bladder cancer, it is improbable that a single marker can accurately segregate tumors of similar clinico-pathologic phenotypes into distinct prognostic categories. Therefore, as previously shown, combinations of independent, complementary markers may provide a more accurate prediction of outcome compared to a single marker [27,30,31,52,80]. Karam and co-workers [59**]
found that p53, Bcl-2, caspase-3, and survivin display distinct association patterns with tumor stage and grade, lymphovascular invasion, and carcinoma in situ. The death of bladder cancer. The number of simultaneously altered apoptosis markers was an important prognostic indicator for disease recurrence and bladder cancer-specific survival in patients treated with radical cystectomy with only 22 patients (10%) showing normal expression of all four markers. Alteration of fewer than four apoptosis markers did not provide independent prognostic information after controlling for the effects of standard pathologic features; only when all four markers were altered was significance reached. This is explained by the fact that Bcl-2, caspase-3, p53, and survivin have not only not independent roles in the apoptotic pathway.

In agreement with previous studies [30,31], Shariat et al. [36–38] confirmed that analysis of any combination of cell cycle regulators (i.e., p53, pRB, p21, p27, cyclin D1, or cyclin E1) provides additional prognostic information beyond that obtained from any single biomarker or combination of two or three biomarkers. This makes sense because it investigates multiple pathways including downstream effector pathways rather than a single crossroad in a pathway. A higher total number of altered biomarkers was associated with a progressive, proportional increase in the risk of advanced pathologic tumor stage, lymphovascular invasion, lymph node metastases, disease recurrence, and death of bladder cancer.

High-throughput transcript profiling

High-throughput genomic and proteomic assays, with appropriate databases and bioinformatics support will be effective tools to screen for novel markers and to fingerprint each cancer with the promise of individualized patient therapy.

Dyrskjot et al. [81] used high-density oligonucleotide microarrays (59,619 genes and expressed sequence tags) to identify a 45-gene signature of disease progression in a training set of 29 bladder cancer patients. An independent test set (74 superficial tumor samples) using house-fabricated 60-mer oligonucleotide microarrays revealed a statistically significant correlation between classifications and clinical outcome. Differentially expressed genes were involved in regulating apoptosis, cell differentiation, and cell cycling and hence may represent potential therapeutic targets.

Blaveri et al. [82] used cDNA microarrays (10,368 genes) to identify differentially expressed genes associated with progression in 80 bladder tumors, nine bladder cancer cell lines, and three normal bladder samples. Unsupervised hierarchical clustering successfully separated the samples into two subgroups containing non muscle-invasive versus muscle-invasive tumors, with a 90.5% success rate and confirmed in an independent tumor set. Tumors could also be classified into transitional versus squamous subtypes (89% success rate) and good versus bad prognosis (78% success rate). Immunohistochemistry validation on tissue microarrays was performed for c-epsin E, cyclin A2, and parathyroid hormone-related protein.

Sanchez-Carbayo et al. [83**] used oligonucleotide arrays to identify genetic signatures characteristic of aggressive clinical behavior in advanced bladder tumors by transcript profiling of 52 normal urothelium, 33 nonmuscle invasive, and 72 muscle invasive tumors. Unsupervised clustering classified them with 82.2% accuracy, while predictive algorithms rendered an 89% correct rate for tumor staging using genes differentially expressed between nonmuscle invasive and muscle invasive tumors. Accuracies of 82 and 90% were obtained for predicting overall survival when considering all patients with bladder cancer or only patients with muscle invasive disease, respectively. Target validation of synuclein by immunohistochemistry on tissue arrays \((n = 294)\) validated its association with tumor staging and outcome.

Aaboe et al. [84*] performed expression profiling of 166 bladder tumor samples and found a fivefold increased expression of human transcription factor SOX4. SOX4 protein overexpression by immunohistochemistry on a tissue array \((n = 2360)\) was correlated with increased patient survival. Overexpression of SOX4 in the bladder cell line HU609 strongly impaired cell viability and promoted apoptosis. SOX4-induced genes are involved in signal transduction (MAP2K5), angiogenesis (NRP2), and cell cycle arrest (PIK3R3).

Other studies using cDNA arrays confirmed genetic signatures including FGFR3 or AIG1 as associated with Ta disease with significant upregulation of 34 genes associated with muscle invasive bladder cancer, including VEGFC or MMP7 and others related to extracellular matrix degradation, immune responses, cell cycling, and angiogenesis [85].

Takata et al. [86] examined expression profiles of 27,648 genes in 27 cases of bladder cancer to predict response to neoadjuvant methotrexate, vinblastine, doxorubicin, and cisplatin (MVAC) chemotherapy regimen. They identified 14 genes with a correct prediction in eight out of nine patients.

Theodorescu et al. [87*] used capillary electrophoresis coupled to electrospray ionization time-of-flight mass spectrometry and bioinformatic tools to obtain polypeptide patterns from urine samples of 46 patients with urothelial carcinoma and 33 healthy volunteers. They
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identified and validated three patterns of polypeptide expression in urine: a bladder-carcinoma pattern, a non-malignant-disease pattern, and a non-bladder cancer–cancer pattern. The biomarkers that constitute these patterns could improve the sensitivity and specificity of bladder cancer diagnosis.

Conclusion

Molecular medicine holds the promise that clinical outcomes will be improved by directing therapy toward the growth of an individual patient’s tumor. High-throughput technologies facilitate comprehensive identification of molecular targets and biomarkers specific for bladder cancer. Such identification processes provide a better understanding of the biology associated with tumorigenesis and tumor progression and identify relevant targets for novel drug therapy and even disease detection. The challenge remains to optimize the measurement of these targets and to translate this wealth of discovery into clinical management of bladder cancer patients.

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This is an excellent article. The presented data thus suggests the existence of two types of bladder cancer. The first consisting of low-grade tumors characterized by FGFR3 activity, either by FGFR3 mutation or by expression, high protein synthesis and low cell-cycle activity. In contrary, the second group show less, or no, dependence of the FGFR3 receptor, low levels of protein synthesis and high cell-cycle gene activity. The presented data thus suggest that low grade/stage bladder cancer are characterized by FGFR3 receptor involvement at a more critical level than previously may have been appreciated.
8 Special commentary

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This describes a very well done study. This study differs, in part, from other published studies on application of array technologies in bladder cancer mainly in that it addresses tumor progression issues of advanced bladder cancer. The authors provide assessment of the significance of the poor outcome profile by means of the Global Test analyses. As part of the clinical relevance of the present approach, the diagnostic ability of novel top-ranked molecular targets was assessed under standard criteria such as ROC curve analyses, and transcript levels cutoffs associated with overall survival were defined. In addition to target identification, the authors present an attempt to delineate allelic imbalances, functional molecular pathways, and signaling networks characteristics of patients with a more aggressive clinical behavior, on the basis of those transcripts differentially expressed on patients with poor outcome. Two independent Global Test runs concluded the robust association or a poor outcome profile with lymph node metastases and overall survival simultaneously.

88 The authors provide evidence for the existence of numerous downstream target genes of SOX4. They show that the SOX4 transcript is highly upregulated in bladder carcinomas and the encoded protein is expressed in a cancer cell-specific manner. Strong SOX4 protein expression was correlated with increased patient survival and that its action in vivo may be due to its involvement in promoting apoptosis, as evidenced by induction of cell death in vitro.

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92 The authors identified and validated three patterns of polypeptide expression in urine from a cohort containing healthy individuals, patients with urothelial carcinoma, and individuals with genitourinary disease or nonurothelial cancer: an urothelial-carcinoma pattern, a nonmalignant-disease pattern, and a nonurothelial-cancer pattern. The biomarkers that constitute these patterns might improve the sensitivity and specificity of urothelial-carcinoma diagnosis, especially in the context of the many confounding diseases routinely found in the assessment of patients with urological disease.