PERGAMON

European Journal of Cancer 39 (2003) 1501–1510

European Journal of Cancer

www.ejconline.com

Current Perspective

Molecular prognostication in bladder cancer—a current perspective

M.L. Quek[a,*], D.I. Quinn[b], S. Daneshmand[a], J.P. Stein[a]

[a]*Department of Urology, Kenneth Norris Jr. Comprehensive Cancer Center, University of Southern California Keck School of Medicine, 1441 Eastlake Avenue, Suite 7414, Los Angeles, CA 90089, USA*

[b]*Medical Oncology, Kenneth Norris Jr. Comprehensive Cancer Center, University of Southern California Keck School of Medicine, 1441 Eastlake Avenue, Suite 7414, Los Angeles, CA 90089, USA*

Received 22 January 2003; received in revised form 14 February 2003; accepted 18 February 2003

Abstract

The optimal management of bladder cancer depends on the accurate assessment of the tumour's biological potential. Advances in molecular biology and cytogenetics have spurred intense research in identifying and characterising prognostic markers for patients with transitional cell carcinoma (TCC) of the bladder. The molecular changes that occur can be categorised into (1) chromosomal alterations leading to carcinogenesis, (2) cellular proliferation as a result of dysregulation of cell cycle control, and (3) growth control processes such as angiogenesis leading to metastasis. The accumulation of these changes ultimately determines a tumour's clinical behaviour and response to therapy. As the understanding of bladder cancer evolves, novel molecular markers for prognostication will make their way from the research laboratory to the clinical setting with the promise to improve patient care and outcomes.

Keywords: Bladder neoplasms; Tumour markers; Transitional cell

1. Introduction

Transitional cell carcinoma (TCC) of the bladder has traditionally been classified into two groups—superficial and invasive disease—based on histopathological examination and clinical behaviour. Approximately 80% of patients with primary TCC will display a relatively indolent, low-grade tumour confined to the superficial mucosa. Most of these patients are managed with transurethral resection and selective administration of intravesical chemotherapy or immunotherapy. Despite the relatively ''benign'' nature of superficial TCC, the recurrence rate can be as high as 70%, thus necessitating frequent costly long-term follow-up. In addition, up to one-third of recurrent superficial tumours may eventually progress to a higher grade and/or stage. Muscle invasive tumours are diagnosed *de novo* in 15–30% of all bladder cancer patients. Unlike superficial disease, invasive TCC typically displays a highly aggressive behaviour, as exemplified by the fact that 50% of patients undergoing definitive local therapy for invasive tumours relapse with metastases within two years of treatment [1]. Clearly, TCC represents a heterogeneous entity with significant malignant potential.

The optimal management of any cancer requires an accurate assessment of its biological potential. Currently, therapeutic strategies for patients with bladder cancer have relied on histopathological determination of tumour grade and stage as the primary prognostic variables. Although these two conventional factors provide a certain degree of prognostic stratification, in terms of recurrence-free and overall survival [2], there remains a significant amount of heterogeneity within various subgroups. Certainly, the ability to accurately and reliably predict a tumour's true biological potential would facilitate a tailored approach to bladder cancer treatment—thereby selecting certain patients who may benefit from adjuvant therapy, while saving some patients from potentially harmful overtreatment.

Cancer cells are distinguished from normal cells by a number of hallmarks including evasion of apoptosis, self-sufficiency in growth signalling, insensitivity to anti-growth signals, sustained angiogenesis, limitless replicative potential, propensity towards tissue invasion, and

* Corresponding author. Tel.: +1-323-865-3700; fax: +1-323-865-0120.

E-mail address: quekm@hsc.usc.edu (M.L. Quek).

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doi:10.1016/S0959-8049(03)00300-9

metastasis [3]. The molecular and genetic changes in TCC of the bladder can be broadly classified into three interrelated processes: (1) chromosomal alterations, triggering the initial carcinogenic event; (2) tumour proliferation, caused by loss of cell-cycle regulation and derangements in normal apoptotic turnover; and (3) metastasis, in which the initial tumour spreads to distant sites, bringing into play processes such as angiogenesis and loss of cellular adhesion.

The accumulation of these successive genetic alterations, rather than a single genetic event, determines a tumour's phenotype and, ultimately, the patient's clinical outcome. In this review, we will summarise the recent literature on some of the more promising molecular markers for prognostication in bladder cancer and comment on potential clinical applications.

2. Carcinogenesis of bladder cancer

2.1. Oncogenes

Oncogenes are normal cellular genes that can become altered by various genetic insults, such as point and insertional/deletional mutations, translocations, and loss of alleles, resulting in the display of a malignant phenotype, either by the overexpression of the normal gene product or by expressing a protein product with altered function [3]. Overexpression involves the gene amplification or chromosomal translocation of the gene to an area downstream from a powerful promoter. Expression of mutated protein products can also lead to deregulation of cellular growth control mechanisms. Oncogenes believed to be important in human bladder cancer include cH-*ras*, *c-myc* and HER2/*neu*.

2.1.1. *cH*-ras *gene*

Mutations in the H-*ras* gene have been implicated in the development and progression of human bladder cancer. Alterations involving codons 12 and 61 of the *ras* oncogene have been found in up to 20% of bladder cancers [8–10]. Frequently, a single point mutation (G to A) in codon 12 leads to activation of the H-*ras* gene, although other mutations have also been described [10]. A potential prognostic role for the cH-*ras* oncogene has been suggested by Fontana and colleagues [11], where overexpression of the cH-*ras* oncogene was correlated with early recurrence in patients with superficial bladder cancer. However, this prognostic effect has not been borne out in subsequent studies [12,13].

2.1.2. c-myc *gene*

Overexpression of the *c-myc* oncogene has been associated with several human tumours, including bladder cancer [14,15]. The *myc* gene family encodes for nuclear phosphoproteins with DNA-binding activity that are important for regulating cellular proliferation [16]. Regulation of *c-myc* activity occurs through a complex heterodimeric interaction with other proteins, namely Mad and Max [17–19]. Chromosomal translocation and gene amplification lead to deregulation of the *myc* gene family [20], thereby promoting cellular proliferation [15].

The prognostic significance of *c-myc* gene expression in bladder cancer remains controversial. Kotake and colleagues [15] demonstrated that expression of the *c-myc* protein as measured by immunohistochemistry correlates with the nuclear grade of bladder cancer. Lipponen [21] on the other hand, found no relationship between *myc* protein expression and prognosis for patients with TCC. Further study will be required to determine the true prognostic role of *c-myc* gene expression in bladder cancer.

2.1.3. *HER2*/neu

The HER2/*neu* oncogene encodes a transmembrane glycoprotein similar to the epidermal growth factor (EGF) receptor, having tyrosine kinase activity [23] and the ability to stimulate cellular growth [24]. Several studies noted an association between HER2/*neu* expression and higher stage tumours [25–27], tumour progression [28], greater incidence of metastatic disease [29], and reduced overall survival [27]. These findings suggest a possible prognostic role for HER2/*neu* expression. However, other reports have found that HER2/*neu* evaluation provided no additional prognostic information over the traditional histopathological markers of grade and stage for TCC [29,30].

2.2. Tumour-suppressor genes

Utilisation of techniques such as loss-of-heterozygosity (LOH) analysis and comparative genomic hybridisation, has made it possible to identify several potential tumour-suppressor genes in bladder cancer that, when inactivated, lead to the expression of the malignant phenotype. Deletions on the short arms of chromosome 3 (3p) [31] and 8 (8p) have been found in high-grade, muscle-invasive TCC [32]. Takle and colleagues [33] and Wagner and others [34] noted 8p deletions in more than 50% of muscle-invasive bladder cancers, with no deletions occurring in superficial disease.

Deletions of chromosome 9 are the most common chromosomal abnormalities associated with bladder cancer [35]. This alteration may in fact represent an early event in the molecular pathogenesis of TCC, given that deletions of chromosome 9 are found with both superficial and muscle-invasive disease [35]. The sequential deletion of different loci on chromosome 9 may be important in bladder cancer development [36] while at least one study has linked such deletions with

tobacco consumption [37]. Other notable chromosomal deletions have been detected on chromosomes 13 (at the retinoblastoma (*RB*) gene) [38,39] and 17 (at the *p53* gene) [32].

2.2.1. Chromosome 9

The majority of chromosome 9 deletions involve the 9p21 locus (*INK4a*/*ARF* and *INK4b*) which encodes for three distinct proteins—$p16^{INK4A}$, $p14^{ARF}$ and $p15^{INK4B}$ [40]. Each of these proteins act as negative cell-cycle regulators, and are therefore, considered potential tumour-suppressor genes [41–43]. Inactivation of the locus appears to require the homozygous deletion of both alleles, thereby removing all three 9p21-encoded proteins [44–47]. The tumour-suppressor function of the 9p21 locus was demonstrated in *in vitro* transfection studies in which $p16^{INK4A}$ introduction into INK4-deleted bladder cancer cell lines led to growth arrest [48]. Currently, $p14^{ARF}$, $p15^{INK4B}$, and $p16^{INK4A}$ proteins are under investigation as possible markers of malignant potential [49–51].

2.2.2. Chromosome 13

The retinoblastoma (*RB*) gene is thought to play an important role in bladder cancer progression. RB gene mutations are found in 25–30% of bladder tumours [39,52] and loss of heterozygosity at the *RB* locus (13q14) is associated with an absence of *RB* protein expression by immunohistochemical techniques [53]. Despite its potential role in bladder cancer, the large size of the gene does not lend itself easily to standard DNA sequencing or single-strand conformational polymorphism analysis [54]; therefore, specific mutational analyses of the *RB* gene are currently limited to the research setting.

2.2.3. Chromosome 17

A well-recognised chromosomal alteration involves the tumour-suppressor gene at 17p13 (*p53* gene). Olumi and colleagues [55] noted the high frequency of loss of heterozygosity at chromosome 17p in high-grade TCC. Genetic defects in the *p53* locus have been shown to correspond with protein expression of the mutated p53 gene product [56,57].

3. Cell-cycle regulatory pathways

Tumour proliferation depends on the derangement of normal cell cycle progression and control [3]. Cell cycle-associated protein complexes composed of cyclins and cyclin-dependent kinases (Cdks) regulate normal cellular proliferation [50,58]. As previously mentioned, several tumour-suppressor genes and their protein products (p53, pRb, $p27^{Kip1}$, $p16^{INK4A}$ and $p14^{ARF}$) act at the G0/G1 checkpoint of the cell cycle to prevent loss of cell cycle control [40,42,59], and ultimately, tumour progression.

Gene alteration may occur by mutation, deletion or methylation; however, in most cases, phenotypic expression requires the alteration of both gene copies. One gene copy may be inherently altered, followed by an environmental mutagen, or both copies may be affected by two independent somatic events, leading to expression of the altered gene product. One notable exception to this "two-hit model" of carcinogenesis is the tumour-suppressor gene *p53*, in which alteration of only one copy is sufficient to alter function.

3.1. Tumour-suppressor genes

Although implicated in carcinogenesis, recent studies suggest that the interaction of several tumour-suppressor genes leads to alterations in cell cycle regulatory pathways, and more accurately reflects the molecular events responsible for bladder cancer progression.

3.1.1. RB *Gene*

The *RB* gene, located on chromosome 13q14, encodes for a nuclear phosphoprotein that normally acts at the G1/S checkpoint to inhibit cell cycle progression [60,61]. The interaction of the Rb-encoded protein with various cell cycle regulatory proteins allows for normal cellular proliferation, while alterations with these protein interactions can subsequently lead to uncontrolled cell growth. Inactivation of pRb may be induced by cyclins, which catalyse the phosphorylation and hence, inactivation of pRb; alternatively, Cdk inhibitors ($p21^{WAF/Cip1}$, $p16^{INK4A}$, $p27^{Kip1}$), which inactivate Cdk/cyclin complexes, may inhibit pRb phosphorylation [61–64].

Several groups have demonstrated that Rb altered tumours are associated with higher grade and stage bladder cancers [38,53]. Further evidence for this was reported from studies by Cordon–Cardo and colleagues [52] and Logothetis [39], in which loss of Rb immunoexpression was found to be associated with a significantly shorter survival in patients with muscle-invasive bladder tumours.

3.1.2. p53 *Gene*

The *p53* gene, located on chromosome 17p13, encodes for a protein vital to arresting the cell cycle [65]. When DNA damage is detected, the level of p53 protein increases, leading to cell cycle arrest. This necessarily allows for DNA repair and prevents propagation of DNA defects [66]. Mutations in the *p53* gene result in the production of a dysfunctional protein product with a longer half-life than the wild-type protein. Because of this difference in protein longevity, p53-mutated gene products accumulate in the cell nucleus and can be easily detected by immunohistochemical methods.

Esrig and colleagues [67] evaluated p53 nuclear immunoreactivity in 243 patients with invasive bladder cancer treated uniformly with radical cystectomy. Altered p53 expression was associated with a significantly greater risk of disease recurrence and reduced overall survival compared with patients with wild-type p53 expression. In addition, nuclear accumulation of p53 was found to be an independent predictor of disease progression in a multivariate analysis evaluating p53 status, histological grade, and pathological stage. A prospective, randomised, multi-institutional trial is currently underway to determine the impact of chemotherapy in organ-confined bladder cancer based on p53 status.

Lipponen [68] and Sarkis and colleagues [69] also demonstrated that increased p53 nuclear reactivity was an adverse prognostic factor for patients with muscle invasive bladder cancer. Lacombe and others [70] demonstrated that p53 nuclear accumulation predicted recurrence in patients with superficial bladder cancer treated with intravesical BCG therapy. Based on these and other studies, p53 immunoreactivity appears to be a consistent marker for bladder cancer prognostication.

3.1.3. *Combination of* RB *and* p53 *genes*

Given the apparent prognostic value of absent Rb expression and p53 nuclear accumulation in bladder cancer, two independent studies sought to determine whether combining these two markers could better stratify bladder cancer patients [71,72]. Indeed, tumours with alterations in both p53 and Rb are associated with a poorer prognosis compared with tumours with normal wild-type p53 and Rb genes. Tumours with alterations in only one of these genes behaved in an intermediate fashion. These studies suggest an independent, yet synergistic role for both p53 and Rb expression in the progression of bladder cancer.

3.1.4. *Role of* $p21^{WAF/Cip1}$ *expression*

Despite the initial enthusiasm for p53 alteration as a predictor of bladder cancer progression, not all p53 mutated bladder tumours recur or progress. In fact, p53 mediates its effects on the cell cycle through the regulation of $p21^{WAF/Cip1}$ expression [58,65]. Therefore, alterations in p53 may lead to loss of $p21^{WAF/Cip1}$ expression and subsequently unregulated cell growth. However, $p21^{WAF/Cip1}$ expression may also be mediated through p53-independent pathways [73,74], thereby maintaining cell cycle control even in the presence of a p53 mutation.

Stein and colleagues [74] evaluated 101 patients with p53-altered tumours treated with radical cystectomy, and found that loss of $p21^{WAF/Cip1}$ expression (by immunohistochemistry) was associated with higher recurrence rates and decreased overall survival compared with $p21^{WAF/Cip1}$-positive tumours. While several other groups have subsequently questioned the prognostic value of $p21^{WAF/Cip1}$ expression [75,76], these findings suggest that $p21^{WAF/Cip1}$ expression through p53-independent pathways may influence cell cycle control, and that tumours with both p53 alterations and loss of $p21^{WAF/Cip1}$ expression appear to have a poorer prognosis. These patients may be candidates for more aggressive adjuvant therapeutic regimens.

3.1.5. *$p27^{Kip1}$ and cyclins D and E*

Del Pizzo and colleagues [77] evaluated 50 TCC specimens with immunohistochemistry for $p27^{Kip1}$ and cyclin E and found that reduced expression of these factors were evident with increased grade, stage and mortality. A subsequent study of 1842 bladder tumours staged Ta through T3 demonstrated that low cyclin E expression is adversely prognostic, but this effect was not sustained when the effect of stage was included [78]. Several other groups have now published data suggesting that low p27 expression with or without low cyclin E expression is adversely prognostic in bladder cancer [79,80]. Decreased $p27^{Kip1}$ expression is prognostic in a number of cancers including breast [81,82], prostate [83] and non-small cell lung cancer [84] and is usually associated with increased cyclin E expression [50]. Why is there a difference in bladder cancer? Juan and Cordon–Cardo have recently described disruption of the nucleoplasm-nucleolar shuttling of cyclin E in bladder cancer cell lines [85], suggesting that altered intranuclear localisation of cyclin E rather than overexpression may be a distinguishing feature of progressive bladder cancer. This hypothesis remains to be tested. Overexpression of a low molecular weight cyclin E has recently been reported to be a major prognostic factor in breast cancer [86,87]. These data suggest that the link between cyclin E and outcome requires further delineation. Interestingly, loss of $p27^{Kip1}$ expression in superficial bladder cancer correlates with disease recurrence and invasion [88,89], as does low expression of cyclin D1 [88]. In the study by Sgambato and colleagues [88] patients with low cyclin D1, low $p27^{Kip1}$ and high proliferative index measured by Ki67 expression had an extremely high rate of recurrence ($P < 0.0001$). In other cancers, cyclin D1 overexpression has been associated with higher proliferation and increased risk of relapse or disease progression. In contradistinction to most other cancers, cyclin D1 expression falls with bladder cancer progression [90]. Why bladder cancer is different in regulation and prognostic effect of cyclin D1 and cyclin E expression yet similar with regard to $p27^{Kip1}$ remains to be elucidated.

4. Angiogenesis and loss of cell adhesion

Angiogenesis is the process in which new blood vessels are formed from the surrounding established

vasculature. During normal development and physiological repair, this event proceeds in a tightly regulated manner [91,92]. Neoplastic conditions also require angiogenesis (neovascularisation) in order to maintain their malignant growth and metastatic livelihood. Therefore, inhibition of tumour angiogenesis may provide another avenue for therapeutic benefit.

Under most homeostatic conditions, angiogenesis is an infrequent process, controlled by an abundant array of inhibitory signals directed at the endothelium, thereby tipping the balance towards neovascular quiescence. Therefore, within a tumour's microenvironment, the balance between various stimulatory and inhibitory inputs to the endothelial cells determines its ability to induce angiogenesis, thus providing the necessary nutrients for continued growth and eventual metastasis.

Several mechanisms are thought to be involved in tumour angiogenesis, including overexpression of various inducers and loss of endogenous inhibitors [93]. These factors may be produced by the tumour cells themselves or released from the surrounding extracellular matrix and tumour-associated stromal cells, or they may be products of the host inflammatory cells that infiltrate the tumour [94,95].

4.1. Microvessel density

Given the role of angiogenesis in tumour growth and spread, one concept that may provide prognostic information is the "microvessel density" within and around a given tumour. By measuring antibodies to factor VIII and CD34 that recognise immature or new vascular endothelial cells, one is able to quantitate the degree of angiogenesis taking place. Microvessel density counts have been correlated with bladder cancer progression and overall survival [96,97]. Dickinson and colleagues [98] evaluated 45 patients with invasive bladder cancer for a median follow-up of 37 months, and found that the microvessel density count was an independent prognostic indicator of disease progression. Furthermore, patients with an elevated microvessel density count demonstrated a 2.5-fold greater risk of dying.

In a review of 164 patients with invasive TCC treated with radical cystectomy, elevated microvessel density was significantly associated with a worse recurrence-free and overall survival [96]. Microvessel density was also noted to be an independent prognostic indicator of disease progression and overall survival when evaluated with histological grade, pathological stage and regional lymph node involvement.

4.2. Angiogenic inducers

Several proangiogenic factors with a potential role in bladder cancer have been identified. Basic fibroblast growth factor (bFGF) has been found in high levels in the urine of patients with bladder cancer, compared with control patients with no bladder cancer [99,100]. Bochner and colleagues [101] also demonstrated that urinary bFGF levels were correlated with pathological stage in patients with muscle-invasive bladder cancer.

Vascular endothelial growth factor (VEGF) is present in higher concentrations in the urine of bladder cancer patients than controls. Furthermore, VEGF levels were correlated with tumour recurrence in patients with Ta and T1 disease [102]. Williams and colleagues [103] also demonstrated high levels of VEGF in the urine of patients with high-grade and/or muscle-invasive TCC compared with those with prostate cancer or no malignancy. In those patients undergoing radical cystectomy, higher preoperative urinary VEGF was associated with a lower 3-year survival.

Increased cyclooxygenase-2 (COX-2) expression has been the focus of considerable interest as a prognostic marker because of the potential to specifically target this pro-angiogenic molecule with inhibitors [107–112]. One study of 108 patients treated with radical cystectomy found detectable COX-2 expression in 31% of cases with expression correlating with local invasion, lymphovascular space involvement and recurrence, but not independently with relapse when local invasion and lymph node involvement were factored into the analysis [113]. Trials of COX-2 inhibitors as preventative and therapeutic agents in bladder and other cancers are ongoing [114,115]. These angiogenic factors clearly play a role in bladder tumour development and growth, although a multitude of inducers are likely to be involved in this process.

4.3. Angiogenic inhibitors

Although several endogenous inhibitors of angiogenesis exist, thrombospondin-1 (TSP-1) has been the most thoroughly examined in human bladder cancer. Campbell and others [104] found that normal urothelial cells contained high levels of TSP-1, and that angiogenesis, induced by VEGF and bFGF could be inhibited by TSP-1, then again reversed by a neutralising antibody.

Grossfeld and colleagues [116,117] demonstrated that low TSP-1 expression was associated with higher recurrence rates and shorter overall survival in patients with invasive bladder cancer. This correlation was strongest in patients with organ-confined disease. In addition, TSP-1 expression was an independent predictor of disease recurrence and overall survival in multivariate analyses. In this same cohort of patients, tumours with a low TSP-1 expression showed higher microvessel density counts [117].

4.4. Extracellular matrix and metastasis

Metastasis depends on a tumour's ability to invade and thrive in the stroma of a distant site [3]. The

complex interaction between stroma and epithelial components, as well as proteolytic-degradation of extracellular matrix by tumour-associated factors undoubtedly plays a role in a tumour's metastatic potential. The extracellular matrix provides the scaffolding for endothelial attachment and subsequent capillary formation. Bladder cancer cells have been shown to induce the production of the angiogenesis inducer scatter factor by the underlying stromal cells [118]. Matrix metalloproteinases are also intimately involved in tumour-associated degradation of the extracellular matrix. Two of these factors, MMP-2 and MMP-9, have been found to be elevated in the serum and urine of patients with muscle-invasive TCC, and correlate with a decreased disease-free survival. MMP-9 expression is also elevated in TCC compared with normal urothelium, and is directly related to an increasing tumour stage [119].

CD44 is a widely expressed cell surface adhesion molecule involved in cell–cell and cell–matrix interactions [120], as well as signal transduction through *ras* in response to hyaluronic acid [121]. Expression of CD44 is increased in superficial TCC with a fall in expression at the time of muscle invasion [122]. This fall in expression is probably due to splice variation in the transcription of CD44 [123, 124]. Recent data suggest that CD44 status is prognostic in urothelial cancer [125]. More specifically, the CD44v6-10 (splice variant) to standard CD44 ratio in urothelial cancer was closely associated with both the presence of tumour and with tumour progression [124–126]. This makes CD44 a potential target for bladder cancer screening.

5. Future directions

The translational application of molecular markers for bladder cancer prognostication continues to evolve. A tumour's ability to grow, invade and spread depends on a multitude of complex interactions that are only now being slowly elucidated at the molecular level. It is unlikely that a single molecular marker will provide adequate insight into a tumour's biological potential. The ultimate application of tumour markers may involve the evaluation of numerous molecular endpoints in a "test battery" approach. This strategy may provide a more accurate assessment of a tumour's phenotype, including responsiveness to both surgical and medical therapeutics. Examples of these include potential molecular markers of response to cytotoxic chemotherapy and/or biological therapy given in a perioperative or maintenance setting. Among the most promising of these are nucleotide excision repair (NER) pathway molecules such as ERCC1 (excision repair cross complementation group 1) [127–133]. Low expression of ERCC1 predicts improved response to and outcome from platinum-based therapy in a number of malignancies including ovarian [134–136], oesophageal [137], gastric [7] and non-small cell lung cancers [138,139]. Targeting of this pathway with response modulators may increase sensitivity to platinum compounds in patients with high levels of NER pathway molecules and who, on this basis, would normally be chemo-resistant [140,141]. Other agents or modalities in which tissue gene expression might be able to predict response in bladder cancer include gemcitabine [142], taxanes [143–145], radiation [146], COX-2 inhibitors [114,115,147] and signal transduction molecules such as epidermal growth factor receptor and HER2/*neu* [148].

Currently, the conventional histopathological assessment of grade and stage allows for only a gross stratification of clinical outcomes for patients with bladder cancer. Despite significant progress in the molecular understanding of neoplasia, the promise for accurate predictions of tumour behaviour based on molecular markers is yet to be realised. The recent development of techniques to interrogate tumours for the expression of myriad genes in multiple tissue sites simultaneously with linkage to outcomes data and other clinical parameters promises to deliver a new level of prognostication and prediction for a number of cancers [149–151]. Molecular techniques will continue to evolve, and clinical trials testing the strongest candidate markers will be necessary to bring this basic science understanding of tumour biology to clinical decision-making and patient care.

6. Conclusions

As our understanding of carcinogenesis, regulation of cellular proliferation, and angiogenesis improves, multiple potential molecular markers for prognostication of bladder cancer will be characterised. The ultimate goal is to identify reliable prognostic markers that can accurately predict a tumour's behaviour, as well as its responsiveness to therapy.

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