

# Molecular prognostic factors in bladder cancer

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## INTRODUCTION

Cancer cells are distinguished from normal cells by several hallmarks, including evasion of apoptosis, self-sufficiency in growth signalling, insensitivity to antigrowth signals, sustained angiogenesis, limitless replicative potential, propensity towards tissue invasion and metastasis [1]. The molecular and genetic changes in TCC of the bladder can be broadly classified into three interrelated processes: (i) chromosomal alterations, triggering the initial carcinogenic event; (ii) tumour proliferation, caused by loss of cell-cycle regulation and derangements in normal apoptotic turnover; and (iii) 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 [2]. Herein we summarize recent publications on some of the more promising molecular markers for prognostication in bladder cancer and comment on potential clinical applications.

## THE CARCINOGENESIS OF BLADDER CANCER

### ONCOGENES

Oncogenes are normal cellular genes that can become altered by various genetic insults, resulting in a malignant phenotype, either by overexpression of the normal gene product or by expressing a protein product with altered function [1]. Oncogenes thought to be important in human bladder cancer include *cH-ras* and *HER2/neu*.

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 39% of bladder cancers [3]. A potential prognostic role for the *cH-ras* oncogene was suggested by Fontana *et al.* [4], where overexpression of the *cH-ras* oncogene was correlated with early recurrence in patients with superficial bladder cancer. Complete loss of p53 is a prerequisite for collaborating with *cH-ras* to promote bladder cancer [5].

The *HER2/neu* oncogene encodes a transmembrane glycoprotein similar to epidermal growth factor (EGF) receptor, having tyrosine kinase activity [6] and the ability to stimulate cellular growth. Several studies noted an association between *HER2/neu* expression and higher stage tumours [7], tumour progression, greater incidence of metastatic disease and reduced overall survival.

### TUMOUR-SUPPRESSOR GENES

Deletions of chromosome 9 are the most common chromosomal abnormalities associated with bladder cancer. Given that deletions of chromosome 9 are found with both superficial and muscle-invasive disease, this alteration may represent an early event in the molecular pathogenesis of TCC [8]. Other notable chromosomal deletions have been detected on chromosomes 13 (at the retinoblastoma, *Rb*, gene) and 17 (at the p53 gene).

Most chromosome 9 deletions involve the 9p21 locus (*INK4a/ARF* and *INK4b*) which encodes for three distinct proteins, i.e. p16<sup>INK4A</sup>, p14<sup>ARF</sup> and p15<sup>INK4B</sup>. Each of these proteins acts as a negative cell-cycle regulator, and they are therefore considered potential tumour-suppressor genes. Chromosome 9 losses occur early in bladder oncogenesis and before p53 alterations or development of aneusomy [9,10].

On chromosome 13, *Rb* gene mutations are found in 25–30% of bladder tumours and loss of heterozygosity at the *Rb* locus (13q14) is associated with an absence of *Rb* protein expression by immunohistochemical techniques.

On chromosome 17, a well-recognized chromosomal alteration involves the tumour-suppressor gene at 17p13 (*p53*). Olumi *et al.* [11] reported 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.

## CELL-CYCLE REGULATORY PATHWAYS

Tumour proliferation depends on the derangement of normal cell-cycle progression and control. Cell cycle-associated protein complexes composed of cyclins and cyclin-dependent kinases regulate normal cellular proliferation [11]. As previously mentioned, several tumour-suppressor genes and their protein products (p53, pRb, p27<sup>Kip1</sup>, p16<sup>INK4A</sup> and p14<sup>ARF</sup>) act at the G0/G1 checkpoint of the cell cycle to prevent loss of cell-cycle control, and ultimately lead to tumour progression.

Gene alteration may occur by mutation, deletion or methylation, but 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.

### TUMOUR-SUPPRESSOR GENES

The interaction of several tumour-suppressor genes leads to alterations in cell-cycle regulatory pathways. The *Rb* gene, at 13q14, encodes for a nuclear phosphoprotein that normally acts at the G1/S checkpoint to

inhibit cell cycle progression. 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. Functional reduction of Rb is associated with progression of bladder cancer to a more malignant and aggressive behaviour [12]. Further evidence for this was reported from studies by Cordon-Cardo *et al.* [13], in which loss of Rb immunoreactivity was associated with significantly shorter survival in patients with muscle-invasive bladder tumours.

The *p53* gene, at 17p13, encodes for a protein vital to arresting the cell cycle [14]. 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. Mutations in *p53* 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 *et al.* [15] 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 than in patients with wild-type p53 expression, and nuclear accumulation of p53 was found to be an independent predictor of disease progression. A prospective randomized multi-institutional trial is currently underway to determine the impact of chemotherapy in organ-confined bladder cancer based on p53 status.

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 patients with bladder cancer. Indeed, tumours with alterations in both p53 and Rb are associated with a poorer prognosis than 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.

Not all p53 mutated bladder tumours recur or progress. Indeed, p53 mediates its effects on the cell cycle through regulating p21<sup>WAF/Cip1</sup> expression [15]. Therefore, alterations in p53 may lead to loss of p21<sup>WAF/Cip1</sup> expression and subsequently unregulated cell growth.

Stein *et al.* [16] evaluated 101 patients with p53-altered tumours treated with radical cystectomy, and found that loss of p21<sup>WAF/Cip1</sup> expression was associated with higher recurrence rates and lower overall survival than p21<sup>WAF/Cip1</sup>-positive tumours. While several other groups have subsequently questioned the prognostic value of p21<sup>WAF/Cip1</sup> expression, these findings suggest that p21<sup>WAF/Cip1</sup> expression through p53-independent pathways may influence cell-cycle control, and that tumours with both p53 alterations and loss of p21<sup>WAF/Cip1</sup> expression appear to have a poorer prognosis. These patients may be candidates for more aggressive adjuvant therapeutic regimens.

Reduced expression of p27<sup>Kip1</sup> and cyclins D and E correlates with increased grade, stage and mortality in bladder cancer [17]. Several groups have reported data suggesting that low p27 expression with or without low cyclin E expression is adversely prognostic in bladder cancer [18]. Decreased p27<sup>Kip1</sup> expression is prognostic in several cancers, including breast, prostate and nonsmall cell lung cancer, and is usually associated with increased cyclin E expression. Juan and Cordon-Cardo [19] recently described disruption of the nucleoplasm-nucleolar shuttling of cyclin E in bladder cancer cell lines, suggesting that altered intranuclear localization of cyclin E rather than overexpression may be a distinguishing feature of progressive bladder cancer. Overexpression of a low molecular weight cyclin E was recently reported to be a major prognostic factor in breast cancer. Interestingly, loss of p27<sup>Kip1</sup> expression in superficial bladder cancer correlates with disease recurrence and invasion, as does low expression of cyclin D1. Patients with low cyclin D1, low p27<sup>Kip1</sup> and a high proliferative index measured by Ki67 expression had an extremely high rate of recurrence.

#### ANGIOGENESIS AND LOSS OF CELL ADHESION

Angiogenesis is the process by 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 [20]. Neoplastic conditions also require angiogenesis (neovascularization) to maintain their malignant growth and metastatic potential. Therefore, inhibiting 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 [21]. 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.

#### 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 recognize immature or new vascular endothelial cells, it is possible to quantify the degree of angiogenesis taking place. Microvessel density counts have been correlated with bladder cancer progression and overall survival [22].

#### ANGIOGENIC INDUCERS

Several human cancers have high levels of growth factors and their receptors that can be used as potential therapeutic targets. Urothelial tumours overexpress tyrosine kinase receptors such as the receptors for EGF (ErbB-1), vascular endothelial growth factor (VEGF) and Her2/neu (ErbB-2). Systemic administration of inhibitors blocks the growth of bladder cancer and enhances the activity of conventional chemotherapy. Several trials are now ongoing, testing agents such as

herceptin (anti-HER2), IMC225 cetuximab, ZD 1389 Iressa and OSI-774 Tacerva (EGF receptor inhibitors).

VEGF is present in higher concentrations in the urine of patients with bladder cancer than in controls, and VEGF levels are correlated with tumour recurrence in patients with Ta and T1 disease [23]. Williams *et al.* [24] also reported higher levels of VEGF in the urine of patients with high-grade and/or muscle-invasive TCC than in 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. In a series of patients with locally advanced bladder cancer and undergoing cystectomy, expression of VEGF and E-cadherin was strongly related to disease-specific 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 [25,26]. Recent experimental work suggests that COX-2 may reduce the cytotoxic effects of chemotherapy. High expression of COX-2 is associated with shorter survival in patients receiving chemotherapy after cystectomy. Trials of COX-2 inhibitors as preventative and therapeutic agents in bladder and other cancers are ongoing [27].

#### ANGIOGENIC INHIBITORS

Although several endogenous inhibitors of angiogenesis exist, thrombospondin-1 (TSP-1) has been examined most in human bladder cancer. It was shown that normal urothelial cells contain high levels of TSP-1, and that angiogenesis, induced by VEGF and basic fibroblast growth factor could be inhibited by TSP-1, then again reversed by a neutralizing antibody.

Grossfeld *et al.* [28] reported 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 low TSP-1 expression had higher microvessel density counts [28].

#### EXTRACELLULAR MATRIX AND METASTASIS

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. Matrix metalloproteinases (MMPs) are also intimately involved in tumour-associated degradation of the extracellular matrix. Two of these factors, MMP-2 and MMP-9, are elevated in the serum and urine of patients with muscle-invasive TCC, and correlate with decreased disease-free survival. MMP-9 expression was also higher in TCC than in normal urothelium, and directly related to increasing tumour stage [29].

CD44 is a widely expressed cell-surface adhesion molecule involved in cell-cell and cell-matrix interactions, as well as signal transduction through *ras* in response to hyaluronic acid. Expression of CD44 is increased in superficial TCC, with a decrease in expression at the time of muscle invasion. Recent data suggest that CD44 status is prognostic in urothelial cancer [30].

#### CONCLUSIONS

The translational application of molecular markers for bladder cancer prognostication continues to develop. 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.

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 research in the molecular understanding of neoplasia, the promise of accurate predictions of tumour behaviour based on molecular markers is yet to be realized. The recent development of techniques to interrogate tumours for the

expression of a myriad genes in multiple tissue sites simultaneously, with linkage to outcome data and other clinical variables, promises to deliver a new level of prognostication and prediction for several cancers. Molecular techniques will continue to develop and clinical trials testing the strongest candidate markers will be necessary to bring this understanding of the basic science of tumour biology to clinical decision-making and patient care.

#### CONFLICT OF INTEREST

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**Abbreviations:** EGF, epidermal growth factor; Rb, retinoblastoma (gene); VEGF, vascular endothelial growth factor; COX-2, cyclooxygenase-2; TSP-1, thrombospondin-1; MMP, matrix metalloproteinase.