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Prognostic Value of Cell-Cycle Regulation Biomarkers in Bladder Cancer

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Abstract

The determination of prognosis in bladder cancer is currently based on staging methods that primarily rely on the pathological stage of a tumor with limited objective correlates. The development and progression of bladder cancer involves alterations in several cellular pathways. Dysregulation in markers associated with cell-cycle regulation have been the most extensively examined molecular aberrations in this cancer. Individual alterations of these markers have been associated with disease outcome, with several observations suggesting that their prognostic potential is independent of pathological stage. While many individual molecules in the cell growth receptor signaling, p53 and Rb pathways have been identified, there is a general lack of consensus on which markers can be adopted in the clinical setting. More recent studies have suggested that the combination of markers as concise panels may be more beneficial in determining the degree of aggressiveness of a given tumor and its impending outcome than individual markers alone. This review will discuss alterations in molecules within pathways controlling cell-cycle regulation in the context of bladder cancer, and their impact on patient outcome when examined individually and in combination.

INTRODUCTION

Comprising over 3% of all cancer cases, carcinoma of the urinary bladder represents the ninth most common type of cancer worldwide.[1] There were nearly 386,300 new cases of bladder cancer diagnosed in the world in 2008, and nearly 150,200 patients succumbed to the disease in the same year.[2] The American Cancer Society estimated nearly 69,250 incident cases and nearly 14,990 deaths due to bladder cancer in 2011 in the USA alone.[3] Urothelial carcinoma (UC) represents the most prevalent subtype of bladder cancer,

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although other histological variants such as squamous cell carcinoma are also common in certain parts of the world.[4]

Patients with UC can present as one of two histopathological stages: a majority present with tumors that are confined to the mucosa and do not invade the basement membrane (noninvasive UC, stage Ta), while an important minority of patients present with invasive cancers that have breached the basement membrane (stages T1–4). Mucosa-confined, noninvasive Ta and lamina propria-invasive T1 tumors are often called non-muscle-invasive UC as they do not invade the muscularis propria.[4] Although these tumors can be easily removed by transurethral resection, 15–70% of cases recur after one year.[5] These patients, however, have prolonged survival, and only 10% of these tumors eventually invade the muscularis propria or metastasize.[6,7] Tumors that invade the muscularis propria, the so-called muscle-invasive UC, have a far worse prognosis.[8,9] Although the 5-year survival rate for all stages combined is 82%, the 5-year survival probabilities for localized cancers and distant metastasis are 94% and 6%, respectively. In fact, the median survival time following cancer recurrence in patients with invasive UC is merely 5.6 months.[10] These data underscore the importance of early detection and appropriate therapeutic intervention in UC.

There is strong evidence to suggest that malignant transformation of the bladder urothelium results from alterations in molecular pathways that are otherwise responsible for the maintenance of cellular homeostasis.[4] The genesis of bladder tumors are generally associated with alterations in two molecular pathways: low-grade, noninvasive UCs usually have alterations in the Ras–mitogen-activated protein kinase (MAPK) signal transduction pathway, while carcinoma *in situ* (CIS) and invasive tumors have deregulations in the p53 and retinoblastoma (Rb) pathways.[11] Alterations in these pathways directly perturb regulation of the cell cycle in urothelial cells that gives rise to the malignant phenotype. Well differentiated (low-grade), noninvasive UCs recur frequently and are often multifocal, but seldom invade the muscle; it is debatable whether such noninvasive tumors represent the malignant origin of invasive lesions that may present later. In contrast, high-grade tumors are often associated with CIS elsewhere in the bladder, which is believed to represent the precursor lesion for invasive tumors. Between these two extremes of the malignant spectrum lie high-grade, noninvasive neoplasms.[12] These tumors have a higher recurrence rate and greater risk of invasion. Such high-grade noninvasive UCs may develop in a distinct pathway via urothelial atypia as in the case of development of CIS, but are accompanied by hyperplasia and ultimately the development of a papillary architecture.[13]

While traditional single-marker studies have shed light on the malignant evolution and progression of UC, no individual biomarker has proven suitably powerful to be used clinically as a prognostic tool on its own. Possible reasons for this include limited reproducibility across studies, varying techniques and biological end-points, and limitations of the study cohorts involved. This review focuses on traditional unimarker studies on cell-cycle regulatory molecules in UC, and more recent multimarker analyses within these pathways that improve the prognostic potential of these biomarkers when examined in combination.

INDIVIDUAL MARKER ALTERATIONS

The most extensively investigated cellular process in UC involves the pathways that regulate cell-cycle progression (Figure 1). Alterations in key molecular markers within these pathways are also important predictors of UC outcome and therapeutic response, and they may also act as druggable targets.[14,15]

Cell Growth Receptor Signaling

Several tyrosine kinase-associated receptors at the cell surface and their downstream molecular cascades transmit growth signals from external cues into the nuclei of urothelial cells. Aberrations in these growth factor receptors and/or signals transmitted by them can lead to an abnormal increase in rate of transduction of growth signals, thereby leading to uncontrolled cellular proliferation and oncogenesis. For instance, altered expression levels of vascular endothelial growth factor receptor 2 (*VEGFR2*), a cell-surface tyrosine-protein kinase receptor for the pro-angiogenic VEGF family of proteins, have been associated with Ta tumor progression and presence of nodal metastasis in UC.[16,17]

The epidermal growth factor receptor (EGFR) family consists of four members that homo- or heterodimerize following ligand activation and transmit signals via the Ras–MAPK pathway, regulating cell-cycle progression, mitogenic signaling, and other processes crucial to UC development (Figure 1). EGFR (ErbB-1) and ErbB-2 (Her2/neu) are among the best-studied receptors in this family. Overexpression of these receptors has been documented in UC.[18,19] Increased EGFR expression has been associated with greater likelihood of progression and death due to UC.[20–22] ErbB-2 overexpression and amplification of its encoding gene in primary UC is highly concordant with concomitant overexpression and amplification in corresponding metastatic lymph node deposits.[23] ErbB-2 overexpression has also been associated with worse disease-specific survival.[19,24,25] While *ErbB-2* gene amplification frequency is low in UC in general, it is present in nearly half of all micropapillary carcinomas and correlates with concomitant protein expression, thereby making it an attractive drug target in this UC variant.[26] In addition, the combined expression profile of EGFR and ErbB-2 was reportedly a stronger predictor of outcome than each individual marker alone,[27] although this finding could not be validated.[28]

The high-affinity cell-surface fibroblast growth factor receptor (FGFR) molecules comprise of four (FGFRs 1–4) members. Activating mutations in *FGFR3* have been the most extensively studied alterations in this family. Nearly 70% of low-grade Ta tumors harbor *FGFR3* mutations, and this alteration is strongly associated with the genesis of low-grade papillary tumors.[29–32] *FGFR3* mutations activate the Ras–MAPK pathway. Mutations in the *FGFR3* and *Ras* genes are mutually exclusive, which probably reflects activation of the same pathway by either event.[33] Nearly 80% of grade 1 tumors and Ta tumors have mutations in either the *Ras* or *FGFR3* genes, suggesting that MAPK pathway activation may be an obligate event in most of these cases.

Mutations in *HRAS* have also been observed in exfoliated tumor cells in the urine of patients with low-grade UC.[34] Studies from our group have also shown that *HRAS* is overexpressed in non-progressing Ta tumors compared with those that progress to an invasive phenotype.[17] Activation of MAPK pathway members have also been shown to predict recurrence, progression, and presence of nodal metastasis across all stages of UC.[16,35]

The p53 pathway

Alterations in the p53 pathway have been classically associated with the genesis of invasive UC.[36] Located on chromosome 17p13.1, the *TP53* tumor-suppressor gene encodes for p53 protein, the primary player of the p53 pathway.[37] The protein has been referred to as the ‘guardian of the genome’ due to its importance in human tumorigenesis in general.[38] By inhibiting cell-cycle progression at the G_1-S transition, p53 acts as a key regulator of the process. The protein mediates this control by activating the transcription of $p21^{WAF1/CIP1}$, a cyclin-dependent kinase inhibitor (CDKI).[39] Although UCs most often exhibit loss of a single 17p allele, mutation in the remaining allele can inactivate *TP53*, thereby resulting in

loss of its tumor-suppressor function.[40,41] However, loss of heterozygosity on chromosome 17 occurs during the later stages of UC and is usually associated with a more aggressive phenotype.[42]

p53 normally has a short half-life that prevents its accumulation in the nucleus.[43] However, *TP53* mutations result in an altered protein that is resistant to normal regulatory ubiquitin-mediated degradation. This causes increased intranuclear accumulation of the protein, which can be detected by immunohistochemistry.[44] p53 nuclear immunoreactivity is predictive of outcome, especially in patients with invasive, organ-confined, node-negative UC.[45–47] From a therapeutic standpoint, while conventional chemotherapy has limited benefits in UC patients, and although cisplatin-based combination therapies have shown mixed benefits in the adjuvant and neoadjuvant settings,[48–51] evidence suggests that patients with locally advanced UC who harbor p53 alterations respond beneficially to cisplatin-based adjuvant chemotherapy.[52] The hypothesis is that DNA damage to p53-altered urothelial cells may result in uncoupling of the S and M phases of the cell cycle, resulting in apoptosis.[53] This was the basis of the international multicenter p53-targeted clinical trial to identify organ-confined invasive UC patients with the greatest risk of progression (i.e., patients with p53-altered tumors) who would respond best to cisplatin-containing chemotherapy.[54] However, the trial could not confirm the prognostic value of p53 nor the benefit of cisplatin-based chemotherapy in UC patients with p53-altered tumors as detected by immunohistochemistry due to high patient refusal rates, lower than expected event rate, and failures to receive assigned therapy that compromised the study's power.

Moreover, while p53 nuclear accumulation as detected by immunohistochemistry has been correlated with *TP53* mutations,[55,56] a significant discordance does exist. Studies comparing p53 immunoreactivity with corresponding *TP53* mutations in primary UC suggest that although both nuclear accumulation and gene mutations are independently prognostic, UCs with mutated *TP53* and an altered protein phenotype exhibit the worst prognosis and those with a wild-type gene and an unaltered protein perform the best.[57] The site of mutation on the *TP53* exome may also be an important prognostic factor.

Located on chromosome 6p21, the $p21^{WAF1/CIP1}$ gene encodes for p21, a CDKI that is regulated by p53, although it can also be modulated in a p53-independent manner (Figure 1). Loss of p21 expression is a possible mechanism by which p53 alterations influence tumor progression. Loss of p21 expression is an independent predictor of UC progression; maintenance of its expression can abrogate the deleterious effects of altered p53.[58] UC patients with p53-altered/p21-negative tumors have a higher probability of recurrence and worse survival compared with those with p53-altered/p21-positive tumors, irrespective of their tumor grade or pathological stage. This is especially notable in node-negative patients.

Encoded on chromosome 12q14.3-q15, the Mdm2 protein is involved in an autoregulatory feedback loop with p53, thereby controlling its activity (Figure 1).[59] Increased p53 levels transactivate the *MDM2* promoter, causing its upregulation. The translated protein then mediates proteasomal degradation of p53. The resultant lowered p53 levels then reduce the levels of Mdm2. *MDM2* amplification has been noted in UC, and its frequency increases with increasing tumor stage and grade.[60] A single nucleotide polymorphism (SNP) in the *MDM2* promoter region, SNP309, has been associated with younger age of disease onset and poorer survival, and it can provide an enhanced prognostic value when combined with *TP53* mutation status.[61]

The p14 protein transcriptionally inhibits *MDM2*. The protein is encoded by $p14^{ARF}$, one of the two splice variant isoforms transcribed from the *CDKN2A* locus located on chromosome 9p21. Because $p14^{ARF}$ can be induced by the E2F transcription factor, it represents the

biochemical link between the p53 and Rb pathways.[62] The other splice variant, *p16*^{INK4a}, encodes for the p16 CDKI protein. UroVysion (Abbott Laboratories, Des Plaines, IL), a clinically used diagnostic test for UC using urine specimens employs fluorescence *in situ* hybridization to detect, among other lesions, homozygous deletions of the *CDKN2A* locus.[63] A positive test result after intravesical therapy indicates a four-fold higher risk for developing tumor recurrence.[64] Homozygous *p16*^{INK4a} deletions in superficial UC have been associated with higher recurrence rate, but only those deletions affecting both p16 and p14, which deregulate both Rb and p53 pathways, have been associated with the worst prognosis.[65] While some reports suggest that *p14*^{ARF} inactivation usually occurs by homozygous deletion, and *p16*^{INK4a} is the hotspot for hypermethylation in UC,[66] other studies have observed higher methylation rates for *p14*^{ARF} than *p16*^{INK4a}.[67]

The retinoblastoma pathway

Alterations in the Rb pathway represent another common anomaly in the development of invasive UC. Located on chromosome 13q14, the *RB* gene was first associated with the development of retinoblastoma.[68] The gene's product (Rb) is a nuclear phosphoprotein that plays an important role in several pathways in bladder tumorigenesis, including cell-cycle regulation, senescence, and apoptosis. Although *RB* mutations have been demonstrated in up to 20% of non-muscle-invasive UCs, deletions and dysfunctional mutations are primarily associated with the invasive and more progressive disease phenotypes.[69–72] Analysis of the entire *RB* coding region using polymerase chain reaction and single-strand conformational polymorphism revealed mutations in 36% of samples with muscle-invasive UC.[72] Grossman *et al* have shown that T1 UC patients with abnormal expression of Rb and/or p53 in their primary tumors had significantly higher probability of tumor progression.[73]

The Rb protein regulates cell-cycle progression at the G_1-S transition. In its active, dephosphorylated form, Rb sequesters the E2F transcription factor by directly binding to it (Figure 1).[74] Upon competitive phosphorylation, Rb releases E2F that in turn transcribes genes necessary for DNA synthesis during S phase of the cell cycle.[75] This results in increased cellular proliferation that can be measured by the Ki-67 labeling index, a widely used immunohistochemical marker for determining the proliferative activity of various tumors. Elevated Ki-67 labeling index has been associated with higher probabilities of UC recurrence, progression and disease-specific mortality.[76,77] Phosphorylation of Rb is promoted by cyclin-dependent kinase (CDK) complexes. These complexes are comprised of an activating kinase component and a cyclin component. Negative regulation of Rb phosphorylation is achieved by CDKIs.

Deletion at chromosome 13q is not the only mechanism of *RB* inactivation. Inactivation of the Rb protein is proposed to be an alternate way of suppressing its function. Interestingly, both high and low Rb immunoreactivity in UC correlate with poor outcome compared to normal Rb immunoreactivity.[78] This observation has lead to the conclusion that evidence of the presence of Rb protein, as indicated by positive nuclear immunoreactivity, does not necessarily imply an intact functioning gene. We have previously demonstrated that in UCs with Rb overexpression as determined by immunohistochemistry, Rb inactivation may be caused by constitutive hyperphosphorylation of the protein and is potentially due to loss of p16 and/or cyclin D1 overexpression.[79]

The CDK complexes that phosphorylate Rb include cyclin D1/CDK4/6 and cyclin E/CDK2. Although a rare event in UC, the frequency of CDK4 amplification has been significantly associated with high tumor grade and greater propensity to invade.[60] *CDK4* overexpression has also been noted in high-grade UC by microarray analysis.[80]

Aberrations in cyclins are widely observed in UC. Cyclin D1 overexpression is present in nearly 20–80% of UCs, and immunoreactivity of the protein is associated with disease invasiveness.[81,82] While current data is equivocal on the prognostic value of cyclin D1, a subgroup analysis in a study of 150 UC cases identified increasing positivity as a predictor of better survival and a lower progression rate in muscle invasive disease.[83] Cytoplasmic cyclin D1 immunoreactivity has also been correlated with higher probability of progression in non-muscle-invasive UC.[84] Cyclin D1 overexpression in a mouse model has been correlated with higher levels of the CDKIs p21 and p27.[85] Our analysis has also revealed that expression levels of another member of the cyclin family, cyclin D3, are associated with recurrence in noninvasive UC.[17] A study of cyclin E expression in 226 cystectomy specimens demonstrated decreased immunoreactivity in 55% of primary tumors and 44% of metastatic lymph nodes.[83] Cancer-specific survival in this group of UC patients was significantly correlated with low cyclin E expression levels.

p21, p27 and p16 are the major CDKIs (Figure 1). These proteins negatively regulate CDK complex activity and are normally required for cell-cycle suppression. p27 inhibits G_1-S cell-cycle progression by binding to the cyclin/CDK complexes.[86,87] Low p27 expression has been associated shortened disease-free and overall survival in UC.[88] While some studies have suggested the possibility of p27 being an important prognostic marker in noninvasive UC,[89,90] issues related to fixation artifacts in p27 immunostaining may warrant reinterpretation of these results.[91]

COMBINED MARKER ALTERATIONS

As illustrated above, the genesis of UC involves alterations in several molecules that are normally associated with regulation of the cell cycle. As a corollary, increasing magnitude of aberrations in these pathways, as measured by greater numbers of altered biomarkers, may correspond to poorer prognosis. Indeed, this is the observation of several studies that have examined alterations in multiple molecules involved in cell-cycle progression in UC.[58,92–98] These and other studies have presented evidence that the combination several independent markers can predict clinical outcome more precisely when compared to the analysis of single molecules (Figure 2).[19,21,78,83,89,99–111]

Our group and others have investigated the combined effects of alterations in individual molecules that regulate the cell cycle. By immunohistochemical analysis of 164 cystectomy specimens from patients with muscle-invasive or high-grade superficial UC for p53, p21 and Rb, we were able to identify four distinct groups of clinical outcomes.[95] Patients with no altered determinant had the best 5-year survival (70%) and lowest 5-year recurrence rate (23%), whereas patients with alterations of all three markers showed the worst 5-year survival (8%) and highest 5-year recurrence rate (93%). Furthermore, we demonstrated that the patients with one or two alterations had intermediate risks of recurrence and death. This correlation was maintained even after controlling for tumor stage. A similar study examining the expression of p53, p21, Rb and p16 on 80 patients who underwent bilateral pelvic lymphadenectomy and radical cystectomy for UC suggested that the incremental number of altered markers was independently associated with an increased risk of UC progression and mortality.[94] Alteration of each of the markers was independently associated with disease progression and disease-specific survival. The study indicated that p53 and p21 were the strongest predictors of outcome, suggesting the central roles of these molecules in UC progression.

A study assessing the value of p53, p21, Rb, cyclin E and p27 expression in patients with organ-confined UC concluded that number of altered biomarkers had the highest predictive accuracy for both disease recurrence and cancer-specific mortality.[112] Addition of the

number of altered biomarkers significantly increased the predictive accuracy of nomograms based on the TNM staging system for disease recurrence and cancer-specific mortality by 10.9% and 8.6%, respectively. These and other findings listed in Figure 2 suggest that the number of molecular alterations may be the most crucial determinant of UC outcome, notwithstanding which specific molecular alterations are present in an individual tumor. Such observations of incremental molecular alterations being associated with worse prognosis have also been recapitulated in UC studies that have focused on pathways other than those that contribute towards cell-cycle progression.[35,113]

Figure 2 also indicates that p53 is one of the most extensively investigated prognostic biomarkers by immunohistochemistry in UC. While most retrospective studies report that the molecule is valuable for determination of outcome by univariate and/or multivariate analyses,[19,21,58,78,83,92–100,102,106–109] others have found it less useful.[89,101,103,105,110] Retrospective studies on p53 also suffer from issues related to antibody selection, lack of assay standardization, and varying cut-off values.[114,115] The p53-targeted clinical trial in UC, however, could not confirm the prognostic value of p53 as detected by immunohistochemistry prospectively due to several inherent limitations in the study design and conduct as mentioned previously.[54] Prior discussion based on our published observations also suggests that p53 immunoreactivity may only be partly indicative of the molecule's status in the tumor, and its association with *TP53* genotype may be crucial in determining UC prognosis.[57] Further examination of this interaction between *TP53* genotype and p53 phenotype in primary UC is an ongoing investigation within our group. Nevertheless, alterations in p53 and its associated molecules within the pathways that control cell-cycle progression possibly have a significant impact on UC outcome, especially when considered in combination (Figure 2).

CONCLUSIONS

Bladder cancer is being increasingly recognized as a disease that cannot be treated based on pathological staging alone; management strategies will need to focus on molecular alterations associated with individual tumors. The various molecular events that lead to urothelial tumorigenesis and progression are now increasingly understood, and several of these alterations manifest as deregulations in cell-cycle progression. Over the past years, several candidate members of the cell growth receptor signaling, p53 and Rb pathways have proven to be useful for determining the outcome of patients with UC.

Tumorigenesis is clearly a multistep process, and while determination of individual molecular alterations offers some biological insight, combined assessment of pathway aberrations is essential to truly determine the aggressiveness of the disease process. Multimarker investigations in dysregulations associated with cell-cycle progression have indicated that the burden of molecular alterations in the tumors of individual patients may be more prognostic than having the knowledge of the specific aberrations. Future UC management will be based on the employment of consensus marker panels that will be able to provide accurate predictions of outcome while also indicating the appropriate therapeutic regimens and targets in individual patients.

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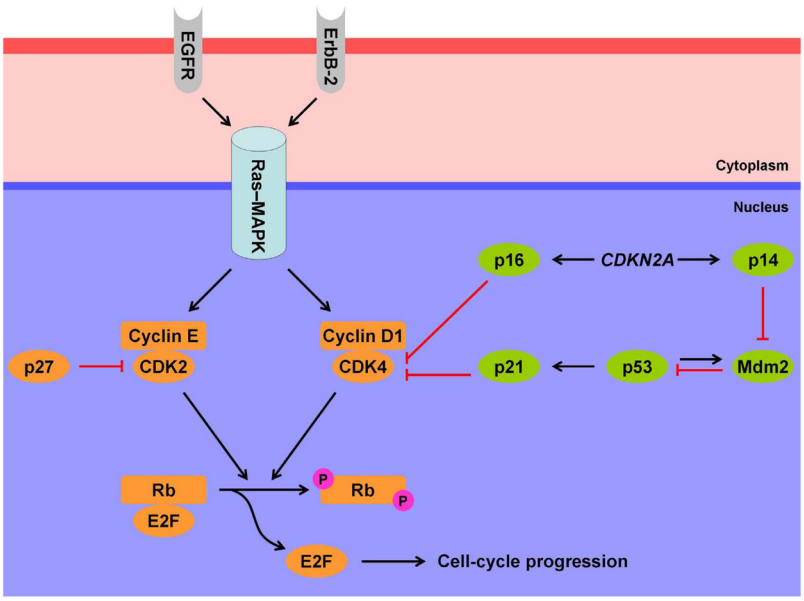

Figure 1. Interactions between cell growth receptor signaling (grey), p53 (green) and retinoblastoma (orange) pathways in controlling cell-cycle progression

Signals from growth receptors on the cell surface are transmitted via the Ras–MAPK pathway into the nucleus, where cyclin/CDK complexes phosphorylate Rb to release E2F, causing progression of the cell cycle and promoting cellular proliferation. This process is regulated by the p53 pathway.

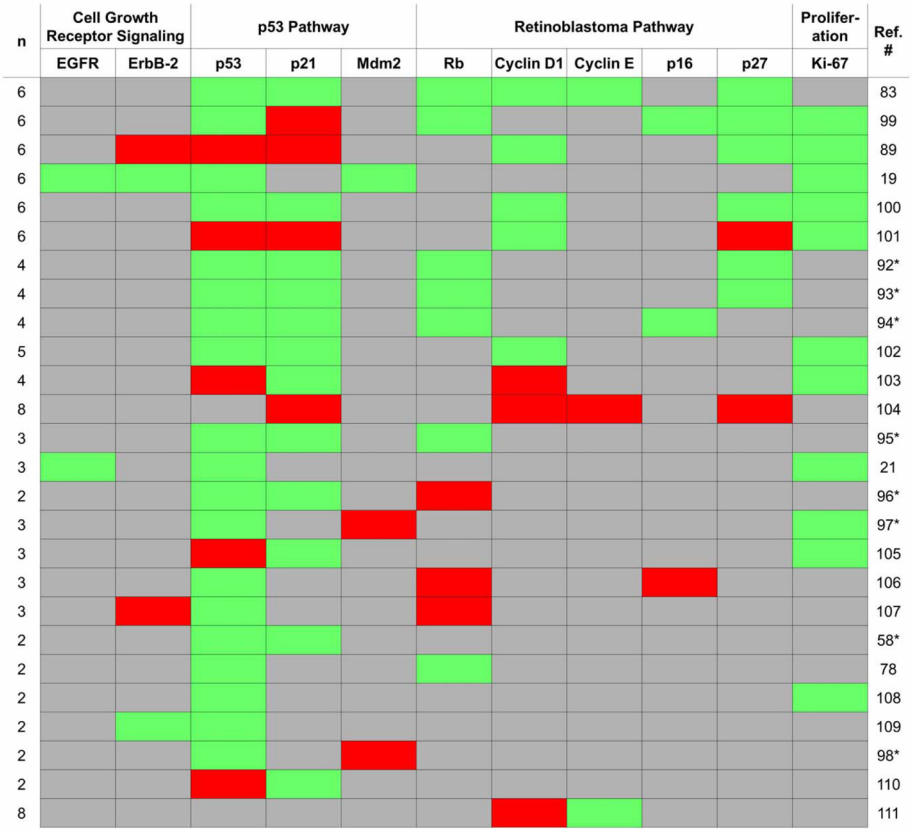

Figure 2. Representative prognostic multimarker studies on molecules involved in cell-cycle regulation in bladder cancer

Individual studies are depicted on rows and referenced in the extreme right column. “n” denotes total number of markers assayed in the study by immunohistochemistry. Green represents significant association with bladder cancer outcome by univariate and/or multivariate analysis; red represents lack of significant association with bladder cancer outcome; grey denotes that the marker was not assayed. * indicates studies providing evidence that increasing number of molecular alterations are associated with worse prognosis.