

Targeted therapies in the management of metastatic bladder cancer

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Abstract: The management of metastatic urothelial carcinoma (UC) of the bladder is a common and complex clinical challenge. Despite the fact that UC is one of the most frequent tumors in the population, long term survival for metastatic disease remains low, and chemotherapy is curative for only a small minority of patients. UC is genetically heterogeneous, and it is surrounded by a complex tissue microenvironment. The problems of clinical practice in the field of metastatic bladder cancer have begun to stimulate translational research. Advances in the understanding of the molecular biology of urothelial cancer continue to contribute to the identification of molecular pathways upon which new therapeutic approaches can be targeted. New agents and strategies have recently been developed which can direct the most appropriate choice of treatment for advanced disease. A review of literature published on the targeted therapy for metastatic bladder cancer is presented, focusing on the molecular pathways shut down by the new therapeutic agents.

Keywords: bladder cancer, metastasis, gene targeting, gene therapy, molecular biology

Introduction

The design and development of agents that act on specific molecular and cellular targets are considered as a rational approach to control cancer. This strategy for control of cancer is based on the presumption that because cancer develops through a multi-step process, each step may be a prospective target for reversing or suppressing the process. There are a number of limitations on drug targeting technology, but, at present, the more difficult limitations are imposed by tumors themselves and by the host's response to a tumor. Moreover, successes *in vitro* are disputable without corresponding data in the more composite organism level.

Bladder cancer is one of the most common cancers, being the 4th most common malignancy in men and the 13th most common malignant cancer in women in the United States (Jemal et al 2007). In 2007, it is estimated that 67,160 new cases of bladder cancer will be diagnosed, and 13,750 deaths will be attributed to this disease (Jemal et al 2007). The incidence is higher in males (with a ratio of 3:1) and in the elderly (Jemal et al 2007). Urothelial carcinoma (UC) (previously designated as transitional carcinoma or TCC) accounts for approximately 95% of bladder malignancies (Baffa et al 2006).

Despite undergoing surgery with curative intent, a large proportion of patients with UC will develop metastatic disease while others will have metastases at the time of initial presentation (Calabro and Sternberg 2006). Accurate clinical staging of bladder cancer remains difficult and inaccurate, with pathologic upstaging after radical cystectomy commonly demonstrated for clinically localized tumors (Ficarra et al 2005). Recent studies have demonstrated that combination therapy with neoadjuvant chemotherapy followed by radical surgery for muscle-invasive diseases offers a small but definite survival advantage (Grossman et al 2003; Vale 2005). Once metastatic,

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however, the relative 5-years survival rate is 6%, whereas the overall 5-years survival for all stages is 82% (Mitra et al 2006). For patients with muscle-invasive disease, the most common pattern of metastasis is to regional lymph nodes, but distant spread to lungs, liver, skin and bone is also typical (Raghavan et al 1990). Metastases to abdominal viscera, brain, and meninges are seen less frequently.

Sites of metastatic involvement correlate with response rate and survival and are important predictors of treatment outcome (Parimoo and Raghavan 2000). Patients with lymph node, lung, and soft-tissue metastases have better survival than those with metastases to bone and liver (Geller et al 1991; Loehrer et al 1992). Biopsies of distant metastatic sites are often consistent histologically with UC pattern. However, a significant disparity within these metastatic lesions with respect to growth parameters, ploidy, karyotype, oncogene expression, tumor markers, grade, and histologic features has been demonstrated (Raghavan et al 1990; Geller et al 1991; Loehrer et al 1992).

Patients with metastatic UC are usually treated with systemic chemotherapy (Sternberg et al 1989; Geller et al 1991; Pagano et al 1991; Calabro and Sternberg 2006). For more than two decades, the standard treatment has been combination therapy with methotrexate, vinblastine, adriamycin and cisplatin (MVAC). This regimen is consistently reported to produce a median survival in the range of 13–15 months (Sternberg et al 1989; Calabro and Sternberg 2006). MVAC has significant toxicity, however, primarily neutropenia, neutropenic fever and severe mucositis, which limits its use in the predominantly older bladder cancer population. Combination therapy with gemcitabine and cisplatin (GC) has shown similar efficacy as MVAC, with less toxicity and a much lower toxic death rate (1% vs 3% for MVAC), leading to widespread substitution of GC for MVAC in clinical practice (von der Maase et al 2000). In the search for regimens more active than MVAC, regimens based on gemcitabine, ifosfamide, and/or paclitaxel have attracted considerable interest (Roth et al 1994; Bajorin et al 1998; Redman et al 1998; Vaughn et al 1998), but until now no substantial improvement in survival has been observed. Most of these treatments are based on the pathologic staging of tumors and do not take molecular profiles into consideration. These facts highlight the limited effectiveness of the current therapeutic regimens and that novel treatment strategies are urgently required. Novel targeted therapies hold promise to improve the current results of metastatic bladder cancer treatment. Several trials are ongoing evaluating these new agents alone or in combination with chemotherapy. The integration

of these newer biologic agents should be a primary direction of research with the objective to interfere with multiple aspects of bladder cancer progression.

Molecular events in metastatic bladder cancer

The identification of mutated genes and gene products which are aberrantly expressed in invasive and metastatic bladder tumors permits the design of molecularly targeted therapies. There have been recent major developments in our understanding of the molecular phenotype of bladder cancers (Table 1) (Dimopoulos and Mouloupos 1998; Knowles 2001; Cote and Datar 2003; Baffa et al 2006; Abraham et al 2007). Over the last two decades, scientists have demonstrated that two distinct molecular pathways are involved in the genesis of UC based on histopathology and clinical behavior (Figure 1) (Wu 2005; Baffa et al 2006): superficial papillary and invasive non-papillary bladder tumors.

Papillary carcinoma, which account for more than 80% of bladder tumors, has a tendency to recur locally (approximately 70%), but rarely invades and metastasizes. On the other hand, most invasive bladder tumors have no known papillary precursor, are solid invasive lesions, are commonly associated with carcinoma in situ (CIS) and have a much less favorable prognosis. Genetic analyses have shown that CIS exhibits a spectrum of genetic alterations (such as *TP53* mutation and loss of heterozygosity -LOH- at 3p, 8p, 13q, and 17p) similar to that seen in invasive UC and very distinct from that seen in low grade papillary UC, where only LOH at chromosome 9 is common (Wu 2005).

In invasive and metastatic bladder cancers, among the different oncogenes and tumor suppressor genes (TSG) which have been studied (Table 1), particular interest has been focused on defects in pathways controlling the G1/S cell cycle checkpoint (involving the tumor suppressor genes *TP53* and *RBI*), angiogenesis, DNA methylation, multidrug resistant genes and on activation of the Ras-MAPK signal transduction pathway, in which associations between molecular abnormalities and tumor prognosis have been identified (Knowles 2001).

Cell cycle regulators

A prerequisite for normal cell proliferation is an orderly progression through the cell cycle, which is predominately controlled by protein complexes that are composed of cyclins and cyclin-dependent kinases (CDK). These complexes control progression through the cell cycle by phosphorylating key proteins that are involved in cell cycle transition points.

Table 1 Principal genes altered in urothelial bladder carcinoma

| Gene | Alteration | Clinical association (References) |
|-------------------------------|-------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Tumor suppressor genes | | |
| FHIT (3p14.2) | LOH, methylation | Survival, muscle invasion (Baffa et al 2000; Skopelitou et al 2001; Vecchione et al 2004) |
| CDKN1A (6p21) | Down-regulation, point mutation | Recurrence, survival, response to chemotherapy (Shariat et al 2004; Stein, Ginsberg et al 1998) |
| FEZ1/LZTS1 (8p22) | LOH, point mutation | Progression (Vecchione et al 2002) |
| CDKN2A (9p21) | LOH, methylation, point mutation | Recurrence (Orlow et al 1999) |
| PTCH (9q22) | LOH, point mutation | – (McGarvey et al 1998) |
| TSC1 (9q34) | LOH, point mutation | – (Hornigold et al 1999) |
| PTEN (10q23) | LOH, point mutation | – (Aveyard et al 1999; Cairns et al 1998; Wang et al 2000) |
| CDKN1B (12q12) | Point mutation | Recurrence, progression, metastatic disease, survival (Franke et al 2000; Kamai et al 2001; Korkolopoulou et al 2000; Lacoste-Collin et al 2002; Sgambato et al 1999) |
| RBI (13q14) | LOH, point mutation | Progression, muscle invasion, survival (Cairns et al 1991; Cordon-Cardo et al 1992; Grossman et al 1998; Logothetis et al 1992; Xu et al 1993) |
| TP53 (17q14) | LOH, point mutation | Progression, muscle invasion, survival, response to chemotherapy (Cote et al 1997; Esrig et al 1993; Lipponen 1993; Lu et al 2002; Pfister et al 1998; Pfister et al 1999b; Sarkis et al 1993, 1995; Sidransky et al 1991; Soini et al 1993) |
| NF-1 (17q11) | Unknown | – (Aaltonen et al 1999; Uchida et al 1995) |
| BCI10 (20q11.1-q12) | Unknown | Muscle invasion (Gromova et al 2002) |
| Oncogenes | | |
| FGFR3 (4p16) | Activating mutation | – (Cappellen et al 1999; Sibley et al 2001) |
| EGFR (7q11.2-q12) | Amplification | Progression (Chow et al 1997; Lipponen and Eskelinen 1994; Mellon et al 1995; Messing 1990; Neal et al 1990; Ravery et al 1997; Sriplakich et al 1999; Vollmer et al 1998) |
| C-MYC (8q24) | Amplification | Progression (Christoph et al 1999; Mahdy et al 2001; Sardi et al 1998) |
| H-RAS (11p15.5) | Activating mutation | Recurrence (Fitzgerald et al 1995; Fontana et al 1996) |
| CCND1 (11q13) | Amplification, chromosomal rearrangements | Progression (Proctor et al 1991; Shin et al 1997; Watters et al 2002) |
| MDM2 (12q13-14) | Amplification | Progression, survival (Habuchi et al 1994; Lianes et al 1994; Schmitz-Drager et al 1997; Shiina et al 1996; Shiina et al 1999; Uchida et al 2002) |
| ERBB-2 (17q21.1) | Amplification | Metastatic disease, survival (Chow et al 2001; Coombs et al 1991; Gandour-Edwards et al 2002; Jimenez et al 2001; Mellon et al 1996; Moriyama et al 1991; Underwood et al 1995) |
| STK15/BTAK (20q13) | Amplification | Metastatic disease, survival (Sen et al 2002) |

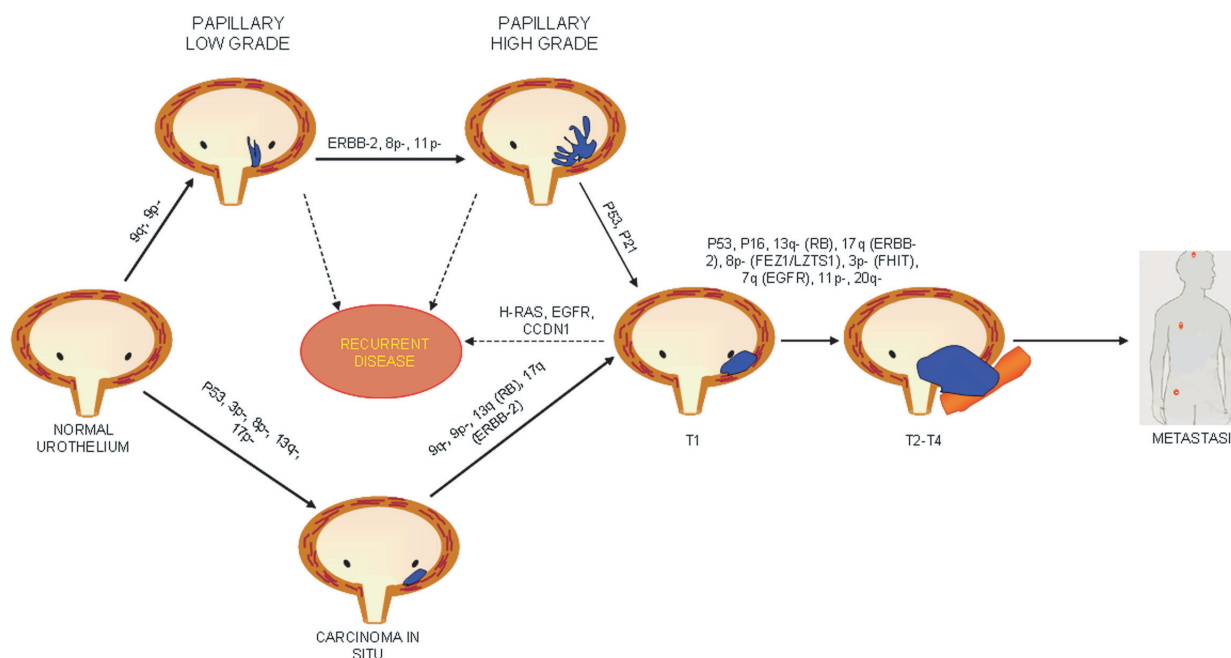


Figure 1 Proposed model for urothelial tumorigenesis and progression. Papillary tumors and carcinoma in situ (CIS) have unique molecular profiles and arise from two distinct pathways.

The fundamental and best studied genes involved in cell cycle regulation are the tumor suppressor genes *RBI*, *TP53*, *CDKN2A* (*P16/INK4A-ARF*) and *CDKN1A* (*P21WAF1/Cip1*) and the oncogene *MDM2*.

The p53 protein is a central molecule in several important cellular programs related to cancer development, progression and response to therapy, as apoptosis and DNA repair (Cote et al 1997). It inhibits cell cycle progression at G1-S transition and mediates its control through the transcriptional activation of *CDKN1A*. *TP53* mutations are the most common genetic defect in human tumors (Hollstein et al 1991) and most studies on *TP53* have used immunohistochemical detection of the p53 protein as a surrogate for gene inactivation by mutation. Mutant p53 has an increased half life and can be easily detected, whereas normal physiological concentrations of the wild-type protein are undetectable. The mutations are generally missense point mutations, which result in altered proteins that are resistant to normal regulatory degradation by the ubiquitin pathway (Dowell 1995). In bladder cancer, mutation of p53 is a feature of more advanced, poorly differentiated tumors and appears to be associated with a high risk of metastatic recurrence and a poor prognosis (Esrig et al 1993; Lipponen 1993; Sarkis et al 1993, 1995; Soini et al 1993; Esrig et al 1994; Pfister, Flaman et al 1999; Pfister, Moore et al 1999). *TP53* has been evaluated in bladder cancer in order to predict and to be correlated with an increased chemosensitivity (McKnight et al 2005; Stein, Grossfeld

et al 1998). Adjuvant chemotherapy was associated with a decreased risk of recurrence and improvement in survival when given to patients with *TP53*-altered tumors (Cote et al 1997). In a transfected bladder cell line, the re-expression of a wild-type p53 protein suppressed the cytotoxic effects of paclitaxel and gemcitabine (Kielb et al 2001). Paclitaxel was shown to require functionally mutated *TP53* to induce cell death, indicating that it may be more effective against UC with *TP53* mutations, while gemcitabine was effective regardless of p53 protein function. Induction of *TP53* gene expression has been shown to be facilitated by prior exposure to cytotoxic agents such as cisplatin and mitomycin C (Parimoo and Raghavan 2000). This altered expression of *TP53* may correlate with increased resistance to combination chemotherapy protocols (ie, MVAC) (Cote et al 1997; Sarkis et al 1995) and may be associated with previous intravesicular treatment (Bajorin et al 1998). All these findings may provide a rationale for selecting chemotherapy on the basis of the *TP53* status.

However, not all bladder tumors with *TP53* alterations progress or recur (Esrig et al 1993; Esuvaranathan et al 2007). As previously described, the action of wild type *TP53* on cell cycle regulation is mediated, in part, through up-regulation of *CDKN1A* (*P21/WAF1*). p21 protein, a CDK inhibitor, binds and inhibits cdk2, a protein that is necessary for transition into the next phase of the cell cycle. Alterations of *TP53* result in loss of p21 expression, which leads to unregulated

cell growth. However, it has been shown that p21 expression can also be regulated through p53-independent pathways which may maintain p21 expression despite the presence of altered p53 (Kinoshita et al 1997). Thus, from a theoretical point of view, p21 protein detection should provide additional information to p53 positivity alone. In bladder cancer, the loss of p21 expression can be a significant and independent predictor of UC progression, whereas the maintenance of p21 expression appears to abrogate the deleterious effects of *TP53* alterations (Stein, Ginsberg et al 1998). In multivariate analysis, p21 labeling was an independent predictor of tumor recurrence and of survival (Stein, Grossfeld et al 1998). Patients with *TP53*-altered/p21-negative tumors demonstrated a higher rate of recurrence and worse survival compared with those with *TP53*-altered/p21-positive tumors (Migaldi et al 2000; Stein, Ginsberg et al 1998).

MDM2 is another gene correlated with *TP53* expression. In normal cells, *MDM2* regulates *TP53* function by marking p53 protein for degradation via ubiquitin conjugation and inactivating p53 by binding to its transactivation domain (Oliner et al 1992; Wu et al 1993). Increased p53 levels transactivate the *MDM2* promoter causing its upregulation. Amplification of *MDM2* results in the escape from *TP53*-regulated growth control. Nevertheless, the role of *MDM2* in regulating p53 protein levels in UC remains unclear. It is generally agreed that Mdm2 over-expression itself provides no independent prognostic information over clinico-pathological parameters (Schmitz-Drager et al 1997; Shiina et al 1999; Uchida et al 2002). However, the combination of *MDM2* and *TP53* status could determine a higher prognostic power on progression (Schmitz-Drager et al 1997; Shiina et al 1999) and survival (Shiina et al 1999) in bladder cancer patients.

The *RBI* gene was the first tumor suppressor gene identified (Friend et al 1986). In its physiologic active form the nuclear phosphoprotein Rb1 encoded protein inhibits cell cycle progression at the G1 to S checkpoint by binding to a number of cellular proteins including the transcription factor E2F (Chellappan et al 1991). The inactivation of *RBI* has been described as an important step in carcinogenesis and progression of various tumors (Bagchi et al 1991; Goodrich 2006). The comparison of immunohistochemical Rb1 expression and molecular analysis in primary UCs showed that *RBI* alterations (observed in the 19%–29.4% of the tumors) are more frequently seen in muscle-invasive and high-grade tumors (Cordon-Cardo et al 1992; Cordon-Cardo et al 1997; Xu et al 1993), and that in patients with muscle-invasive bladder cancer the 5-year survival was significantly decreased

in cases with altered Rb1 protein (Cordon-Cardo et al 1992; Logothetis et al 1992; Gallucci et al 2005).

Important in the regulation of the cell cycle is also the *CDKN2A* gene (also known as *P16*, *INK4A*, *MTS1*, *CDKN2A*), which is a well-characterized CDK inhibitor (Lo et al 1996). This protein functions upstream of Rb1 to block cyclin-D directed phosphorylation of Rb1, thus inducing G1 arrest. *CDKN2A* mutations and homozygous deletions have been detected in the 3.1% and 14.1% of bladder cancers analyzed, respectively (Orlow et al 1999). Furthermore, *CDKN2A* is thought to be susceptible to transcriptional silencing by promoter methylation, which affects from 14.9% to 26.5% of the tumors tested (Orlow et al 1999; Chan et al 2002). Inactivation of *CDKN2A* by any of the mechanisms will lead to affect both p16 and p19ARF proteins, and subsequently disrupting the Rb1 and p53 pathways (Orlow et al 1999).

The interaction of p53 and p21 proteins in cell cycle regulation, and the data looking at the cooperative effects of p53 and Rb1 (Cordon-Cardo et al 1997), provide good examples of the increasing evidence that mutation in a single TSG is unlikely to be the only factor resulting in carcinogenesis. Several studies have conducted multivariate analyses using various combinations of cell cycle regulators markers (*TP53*, *CDKN1A*, *RBI*, *CDKN2A*, *MDM2*) to generate prognostic panels (Lu et al 2002; Shariat et al 2003; Shariat et al 2004). Lu et al (2002) and Shariat et al (2003) found that the 12.1% of the 140 tumor analyzed, had an altered p53 pathway, defined by the detection of mutant *TP53* and/or p53 nuclear overexpression, loss of p21 nuclear expression, and Mdm2 nuclear overexpression. Moreover, they exhibited the worst clinical outcome in the observation period. Shariat et al (Shariat et al 2004), found that the 83% of 80 bladder cancers had at least one marker altered (p53, p21, and pRB or p16), and 21 patients (26%) had all three altered. These studies demonstrated a biological and pathological relationship between these markers spanning multiple molecular pathways and progression, suggesting their important role in bladder carcinogenesis and their rational target for the design of molecularly targeted therapies.

Tumor angiogenesis

Angiogenesis is not only important in maintaining the supply of oxygen and nutrients to the proliferating tumor cells and to the metastatization of the primary tumor, but also for the survival and spreading of secondary localizations. The most important factor affecting this process is the pro-angiogenic vascular endothelial growth factor (VEGF). High serum

VEGF levels and VEGF IHC expression have been associated with high UC stage and grade, vascular invasion, CIS, metastases and poor disease free survival (Bernardini et al 2001; Zu et al 2006). Moreover, VEGF levels affect the production of interleukin-8 (IL-8) and matrix metalloproteinases (MMPs) (Nutt et al 1998), which enhances angiogenesis of the tumor, and migration and invasion of bladder cancer cells. Increased expression of MMP-9, IL-8 and VEGF has been proven to be correlate with stage and outcome of advanced bladder tumors (Black and Dinney 2007).

DNA methylation

The promoter methylation has been demonstrated to be a frequent mechanism of inhibition for important genes including those that influence the cell cycle and signal transduction in cancer (Chan et al 2002). In UC, the role of this epigenetic event in the silencing of TSGs is still under study. The promoter region of *TP53* gene is almost never methylated in muscle-invasive UC (Kunze et al 2006), whereas, as mentioned above, *CDKN2A* is susceptible to transcriptional silencing by promoter methylation (Orlow et al 1999; Chan et al 2002). Recently, additional findings are confirming the importance of this mechanism in the process of bladder metastatization (Nixdorf et al 2004). Further studies should elucidate the significance of hyper-methylation and the role of demethylating agents in the treatment of locally advanced and metastatic UCs (Cote and Datar 2003).

Multidrug resistant genes

The tumor resistance to chemotherapy is the major problem affecting the management of metastatic and aggressive bladder cancers. Over-expression of the *MDR1* gene leads to drug resistance via up-regulation of a membrane-bound 190 kDa phosphoglycoprotein, that serves as an energy-dependent drug flux pump and eliminates toxic metabolites out of the cells (Hasegawa et al 1995). The expression of this protein correlated in various tumors with low local drug accumulation and increased drug resistance (Endicott and Ling 1989), which has also been observed in bladder cancer (Clifford et al 1994; Naito et al 1992; Petrylak et al 1994). Petrylak et al (1994), observed a positive p-glycoprotein immunostaining in 6 of 46 untreated primary lesions (13%), while 6 of 16 (38%) post-therapy primary tumors were immunoreactive. None of the untreated metastases (0 of 17) were positive; however, 6 of 11 (55%) post-therapy specimens showed varied percentages of positivity. Of interest, the highest percentage, 50%–70% of tumor cells stained, was observed in metastatic lesions from patients who had received 6 or

more chemotherapy cycles. These characteristics suggest that targeting of *MDR1* expression may improve the response of advanced and metastatic bladder tumors to systemic chemotherapy.

Growth factor receptors

In invasive and metastatic UC two receptor tyrosine kinases, the epidermal growth factor receptor (*EGFR*) and *ERBB-2* (also called *HER-2* or *NEU*), are over-expressed (35%–86% for *EGFR*, and 41%–52% for *ERBB-2*) and associated with poor outcome (Lipponen and Eskelinen 1994; Ravery et al 1997; Kruger et al 2002; Hussain et al 2007)

Preclinical and clinical data strongly support the involvement of the *EGFR* in the growth and progression of human cancers (Nicholson et al 2001; Mendelsohn 2002; Dei Tos 2007) as well as demonstrate a high correlation in cancer patients between receptor/ligand expression and poor prognosis (Nicholson et al 2001). *EGFR* is a transmembrane glycoprotein that is activated by the binding of epidermal growth factor (EGF) and of transforming growth factor (TGF) to its external domain. In normal cells, binding of EGF causes activation of the *EGFR*, leading to receptor dimerization and autophosphorylation. The activated receptor then recruits proteins that convert Ras to its activated state, which can then transduce a mitogenic signal through the Ras-MAPK pathway by activating the MAPK/ERK complex. This activation sets off several cell regulation processes such as proliferation, migration and adhesion (Messing 1990; Messing 1992; Roberts and Der 2007). Although the mechanism by which *EGFR* regulates tumor biology in bladder cancer is not clearly defined, it has been demonstrated that *EGFR* signaling regulates cellular proliferation, differentiation, survival, invasion; and it is implicated in the induction of tumor induced angiogenesis and metastasis (Mendelsohn and Dinney 2001; Nicholson et al 2001).

Immunohistochemical studies have demonstrated that *EGFR* is over-expressed in human UC compared to the normal urothelium (Neal and Mellon 1992; Rotterud et al 2007). Moreover, it has been observed that normally the urothelial cells which over-express *EGFR* are found primarily in the basal layer of the urothelium (Messing 1990; Messing 1992; Rotterud et al 2007), whereas, in malignant and dysplastic urothelium, *EGFR* is expressed in all cell layers (Baffa et al 2006). Most importantly, it has also been observed that the level of *EGFR* expression directly correlates with tumor grade, stage, and survival (Neal et al 1990; Messing 1992; Lipponen and Eskelinen 1994; Mellon et al 1995; Chow et al 1997; Popov et al 2004). Patients with muscle-invasive UC

which over-express EGFR have only a 20% probability of long term cancer-specific survival, which is significantly worse than the survival of those whose tumors did not express EGFR (Nguyen et al 1994). In metastatic bladder cancer, the majority of metastases from patients over-expresses the EGFR, and this expression is not down-regulated by chemotherapy or radiation (Nguyen et al 1994).

In normal and malignant cells, the preferred partner for the EGFR molecule is erbB-2, a member of the *ERBB* gene family, and encoded by the *ERBB-2/HER-2/NEU* gene. This heterodimeric formation acts as the most efficient receptor for EGF. As observed in breast, gastric and ovarian cancers, the erbB-2 protein was found to be over-expressed frequently in urinary bladder carcinoma (Messing 1992; Sato et al 1992; Roberts and Der 2007) and it has also been found associated with increasing tumor grade, poor survival and incidence of metastatic disease (Moriyama et al 1991; Sato et al 1992; Gandour-Edwards et al 2002). The prognostic power of its over-expression increases when combined with other erbB receptors (especially EGFR and erbB-3) (Chow et al 2001). Of interest, in one study

(Jimenez et al 2001) almost 70% of erbB-2 negative primary muscle-invasive tumors had erbB-2 positive corresponding distant metastasis.

From the bench to the bedside

Advances in the understanding of the molecular biology of UC continue to contribute to the identification of molecular pathways upon which new therapeutic approaches can be designed. The goal of targeted therapy is to optimize the therapeutic ratio of an anti-neoplastic drug by maximizing its effect on tumor cells, and at the same time minimizing toxicity for normal cells. Various approaches have been developed to specifically targeting the phenotype of tumors. Such therapies involve the use of antibodies or small molecule enzyme inhibitors which interact with specifically target molecules differentially expressed between tumor and normal cells. In Table 2 the wide range of novel therapeutic agents (viral vector carrying wild-type genes, small molecule inhibitors and monoclonal antibodies) are summarized. Many of these agents are being introduced in clinical trials.

Table 2 Innovative targeted agents for metastatic bladder cancer treatment

| Target | Agent | Mechanism of action |
|--------------------------------|--------------|-------------------------------------------------------------------------------------------------------------|
| p53 mutated | AdCMV-TP53 | Delivery of functional TP53 into cells (Pagliaro et al 2003b) |
| p53 mutated | rVV-TK-53 | Delivery of functional TP53 into cells (Fodor et al 2005) |
| p53 mutated | ONYX-015 | Delivery of functional TP53 into cells (Khuri et al 2000) |
| p53 wild-type and mutated | CP-31398 | Restores mutant p53; stabilizes wild-type p53 (Ho and Li 2005; Tanner and Barberis 2004) |
| p53 mutated | PRIMA-1 | Restores transcriptional activity of mutant p53 (Bykov et al 2005) |
| Rb-positive and negative cells | Ad-Rb94 | Replaces Rb function (Zhang et al 2003) |
| Cyclin-dependent kinases | Flavopiridol | Nonspecific cyclin-dependent kinase inhibitor (Senderowicz 2003b) |
| Cyclin-dependent kinases | UCN-01 | Nonspecific cyclin-dependent kinase inhibitor (Senderowicz 2003b) |
| EGFR | Gefitinib | Inhibition of tyrosine kinase activity (Ciardiello et al 2000) |
| EGFR | Erlotinib | Inhibition of tyrosine kinase activity (Lindsey 2006; Meyerhardt et al 2006) |
| EGFR | Cetuximab | Prevents signal transduction (Inoue et al 2000) |
| erbB2 | Trastuzumab | Inhibits HER2 and activates anti-tumor immune response (Hussain et al 2007; Sawyers 2002; Small et al 2003) |
| Receptor tyrosine kinase | Sorafenib | Multikinase inhibitor (Carter et al 2007; Kupsch et al 2005; Panka et al 2006; Siu et al 2006) |
| VEGF | Endostatin | Inhibition of cell growth and migration (Kikuchi et al 2004) |
| VEGF | Bevacizumab | Binds and inactivates VEGF (Jain et al 2006) |
| VEGF | VEGF Trap | Binds and inactivates VEGF (Kim et al 2002) |
| VEGF | Sunitinib | Inhibition of tyrosine kinase activity (Silay and Miroglu 2007) |
| VEGF | Pazopanib | Inhibition of tyrosine kinase activity (ClinicalTrials.gov) |
| VEGF/EGFR | ZD6474 | Inhibition of tyrosine kinase activity (Ciardiello et al 2003) |
| Hypermethylated TSG promoters | 5-Aza-CR | DNA incorporated (Mitra et al 2006) |
| Hypermethylated TSG promoters | 5-Aza-CdR | DNA incorporated (Yoo and Jones 2006) |
| Topoisomerase I | Irinotecan | Topoisomerase I inhibition (ClinicalTrials.gov) |
| Histone deacetylase | Vorinostat | Histone deacetylase inhibitor (ClinicalTrials.gov) |
| 20S proteasome | Bortezomib | dipeptidyl boronic acid inhibitor (Calabro and Sternberg 2006) |

Targeting cell cycle regulators

The knowledge that genetic alterations of the *TP53* gene occur in up to 70% of muscle-invasive bladder cancers (Knowles 2001) make *TP53* gene an extremely attractive target for rationally designed therapies. Small molecules that can directly restore *TP53* function are CP-31398 (Foster et al 1999), which should restore the conformational structure and DNA-binding ability of mutant p53, and PRIMA-1 (*P53 Reactivation and Induction of Massive Apoptosis-1*), which has been shown to suppress the growth of cells expressing mutant p53 (Bykov et al 2002) and to synergize with cisplatin to induce tumor cell apoptosis (Bykov et al 2005).

However, the most suitable approach to target p53 pathway, seems to be the direct delivery of wild-type *TP53* gene by viral vector in intra-vesical instillation. The first clinical trial involving intra-vesical delivery of a gene therapy vector (vaccinia virus) has been published by our group (Gomella et al 2001). The vector used has been subsequently recombined with *TP53* (rVV-TK-53) in orthotopic murine animals (Fodor et al 2005). Similar gene therapy trials, in which adenovirus containing *TP53* gene (AdCMV-*TP53*) was used, have demonstrated tumor inhibition in bladder cancer cell lines and xenograft models (Pagliaro, Keyhani, Liu et al 2003). Two similar adenoviral vectors containing the *TP53* gene have been instilled into the bladder, both as single and multiple instillations, and have led to expression of functional p53 that can be detected in the bladder epithelium (Kuball et al 2002; Pagliaro, Keyhani, Liu et al 2003). In addition, these trials involving intravesical instillation of the vector have revealed a high level of tolerance, increased transduction efficacy and expression when used in combination with transduction-enhancing agents (Kuball et al 2002; Pagliaro, Keyhani, Liu et al 2003), and a synergistic effect in combination of cisplatin leading to increased apoptosis (Pagliaro, Keyhani, Williams et al 2003). Pagliaro, Keyhani, Williams et al (2003), observed evidence of tumor response in one of 13 advanced superficial bladder cancers treated.

Another gene therapy viral vector, which might be used in the delivery of a normal *TP53* gene in bladder cancer cells, involves the selectively replicating adenovirus dl1520 (ONYX-015) (Heise et al 1997). Deletion of the E1B 55 kDa protein gene from the viral genome results in selective replication of cells that lack a functional p53 pathway. In normal cells with functional p53, the virus cannot replicate and is therefore harmless (Heise et al 1997; Ries and Korn 2002). In a number of Phase I and Phase II clinical trials, the use of this viral vector has demonstrated a safety record and has been shown to be effective in combination with

cisplatin, as combined-modality therapy (Khuri et al 2000). In a single study, treatment caused tumors to shrink in 25 of the 30 cases evaluated (Khuri et al 2000). Objective responses (decrease of 50% or more) of injected tumors were documented in 63% of patients who could be evaluated (19 of 30). There were 8 (27%) complete and 11 (36%) partial responses. ONYX-015 therefore represents an attractive agent for the treatment of the majority of high-risk *TP53* mutant bladder cancers. Similarly, conditionally replicating E1a-deleted adenoviruses (Hernandez-Alcoceba et al 2000) may selectively target approximately 37% of muscle-invasive bladder cancers which have shown mutations in the *RBI* tumor suppressor gene. In normal bladder cells with functional Rb1, the absence of viral E1a gene function will prevent replication of the viral genome. However, in bladder tumors with mutant Rb1, there should be no effective barrier to viral replication and as with ONYX-015 in p53-defective cells, the virus would be expected to be selectively apoptotic. Another vector carrying *RBI* is Ad-RB94, which is a replication-deficient adenoviral construct with Rb94, a protein which lacks 112 amino acid residues of the wild-type Rb1 protein (Rb110) resulting in a more potent tumor and growth suppressor than the normal protein (Xu et al 1994). This vector has been observed to be very selective to both bladder cancer *RBI*-negative and *RBI*-positive cells, inducing growth suppression and caspase-dependent apoptosis (Zhang et al 2003).

Recent data proposed hyper-phosphorylation of *RBI* as mechanism for Rb1 tumor suppressor pathway inactivation in bladder cancer (Chan et al 2002). Thus, a treatment leading to hypo-phosphorylation of the wild-type *RBI* promoter using CDKIs may improve prognosis. Recently, non-specific CDKIs like flavopiridol (L86-8275) and UCN-01 (7-hydroxystaurosporine) have entered clinical trials (Senderowicz 2003b). Moreover, flavopiridol also decreases cyclin D1 levels, which are elevated in many UC cases (Senderowicz 2003a).

Targeting growth factor receptors

Two of the principal targets in the signal transduction cascade in metastatic and invasive tumors are EGFR and erbB-2 proteins (Bellmunt, de Wit et al 2003; Bellmunt, Hussain et al 2003). A series of studies targeting both the EGFR and the erbB-2, which are overexpressed or amplified in bladder cancer (Bue et al 1998; Gardmark et al 2005; Jimenez et al 2001; Scholl et al 2001; Wester et al 2002), are under way and will help to define the role of these new targeted therapies in the treatment of advanced UCs.

Several strategies have been designed to target these receptor tyrosine kinases (Mendelsohn 2000). The two most studied approaches to targeting EGFR are monoclonal antibodies against the extracellular domain of the receptor (Cetuximab/IMC-C225 [Erbix]) and inhibitors of the receptor tyrosine kinase domain (Gefitinib/ZD1389 [Iressa], Erlotinib/OSI-774 [Tarceva]) (de Bono and Rowinsky 2002; Mendelsohn 2002). Trastuzumab (Herceptin), a monoclonal antibody against erbB-2, has prompted the initiation of a phase I/II clinical trial to determine the toxicity of combined chemo-radiotherapy (paclitaxel, carboplatin and gemcitabine) with or without this agent in patients with prior cystectomies for muscle invasive bladder cancer (ClinicalTrials.gov; Hussain et al 2003; Calabro and Sternberg 2006; Hussain et al 2007). A recently introduced agent, sorafenib (BAY 43-9006 [Nexavar]), is a multikinase inhibitor targeting various molecules, including EGFR and erbB-2, and is currently in phase II clinical trials for advanced and metastatic UC (ClinicalTrials.gov).

Based on the success seen with anti-erb-2 monoclonal antibodies and the promising results with EGFR targeted agents in other tumor types, there is a great interest in assessing these agents in patients with bladder cancer. Inhibition of EGFR and erbB-2 pathways, either by physical receptor blockade or with small molecule inhibitors of the receptor's tyrosine kinase activity, leads to demonstrable anti-tumor effects in animal models (Nicholson et al 2001; Mendelsohn 2002; Hidalgo 2003). More importantly, multiple reports confirm that EGFR directed therapy in combination with cytotoxics produces a much-enhanced biologic effect (Mendelsohn 2002). Blocking signaling through EGFR on tumor cell surfaces can promote apoptosis, inhibit angiogenesis and metastases, and consequently cause tumor regression (Izawa et al 2001).

Gefitinib, as erlotinib, inhibits the activity of tyrosine kinase in the intracellular component of the EGFR, thus preventing receptor autophosphorylation and subsequent activation (Slichenmyer and Fry 2001). The combination with platinum-derived agents (cisplatin, oxaliplatin, carboplatin), taxanes (paclitaxel, docetaxel) and topoisomerase inhibitors, shows enhanced growth inhibition (Ciardiello et al 2000). Its anti-proliferative effect in bladder cancers has been demonstrated in vitro and in vivo, and now it is in phase II clinical trials for advanced or metastatic UC (ClinicalTrials.gov; Dominguez-Escrig et al 2004; Nutt et al 2004). Erlotinib, in combination with green tea extract (Polyphenon E) is under study in a phase II clinical trial for

preventing cancer recurrence in former smokers with resected UC (ClinicalTrials.gov).

Cetuximab binds to the EGFR with high affinity, blocks ligand-induced tyrosine kinase activity and stimulates receptor internalization. In vitro, proliferation of 253J B-V cells was inhibited more by the combination of cetuzimab and paclitaxel than with either cetuzimab or paclitaxel (Inoue et al 2000). The combination enhanced apoptosis in tumor and endothelial cells compared with either agent alone, most likely mediated by inhibition of angiogenesis and induction of apoptosis (Perrotte et al 1999). The combination of cetuximab and paclitaxel has been evaluated in mice with metastatic human bladder UC with encouraging results (Inoue et al 2000).

New therapeutic targets

One of the mechanisms used in cell transformation is the escape from the normal control mechanisms of the apoptotic process. In the attempt to predispose cancer cells to apoptosis, anti-sense oligonucleotide gene therapy directed to *BCL-2* mRNA has already been demonstrated to reverse cisplatin resistance in bladder tumor cell lines in vitro, (Hussain et al 2003) and it will be interesting to see whether these results can be reproduced in pre-clinical models and in clinical trials.

Recently, the importance of VEGF and bFGF in advanced and metastatic UC has grown and a number of agents have been designed against them, many of which have already entered clinical trials. The most popular is endostatin, which decreases VEGF expression and tumor growth by inducing apoptosis (Du and Hou 2003). Moreover, the lentiviral gene transfer of endostatin by intravesical instillation decreases orthotopic human bladder tumor growth (Kikuchi et al 2004). Bevacizumab (Avastin), a monoclonal antibody against VEGF, already used for other type of cancers (Jain et al 2006), is in phase II clinical trials in combination with cisplatin and gemcitabine for metastatic UC (ClinicalTrials.gov). VEGF Trap consists in a fully humanized, soluble decoy VEGF receptor generated by fusing the extracellular domains of VEGFR-1 and VEGFR-2 to the Fc portion of human IgG1. The mechanism of action is very similar to bevacizumab, binding and inactivating VEGF. In preclinical models it inhibits tumor growth and angiogenesis (Kim et al 2002). In addition, it is in use in clinical trials against metastatic UCs (ClinicalTrials.gov).

Bortezomib (PS-341) is a dipeptidyl boronic acid inhibitor of the 20S proteasome, which also inhibits secretion of the pro-angiogenic factors matrix metalloproteinase-9,

interleukin-8 (IL-8), and vascular endothelial growth factor (VEGF) (Zavrski et al 2005). The effects of bortezomib on the growth of human 253JB-V bladder cancer cells showed inhibition of cell growth in a concentration-dependent fashion and higher growth inhibitory effects of gemcitabine in vitro (Calabro and Sternberg 2006). These effects were associated with accumulation of p53 and p21 and suppression of cyclin-dependent kinase 2 activity. In vivo studies with 253JB-V tumors growing in nude mice demonstrated that bortezomib did not inhibit tumor growth when it was delivered as a single agent. However, the combination therapy with bortezomib plus gemcitabine produced synergistic tumor growth inhibition associated with strong suppression of tumor cell proliferation (Calabro and Sternberg 2006).

The role of hyper-methylation of tumor suppressor gene promoters in UC has recently been stressed, highlighting the importance of using demethylating agents to reverse the hypermethylation in advanced and metastatic UCs (Cote et al 2005). The most used agents are nucleoside analogs such as 5-azacytidine (5-Aza-CR), 5-aza-2'-deoxycytidine (5-Aza-CdR, decitabine), and zebularine. These therapeutics have a modified cytosine ring attached to either a ribose or deoxyribose moiety, which inhibits the DNA methylation (Yoo and Jones 2006) and they are now under study in the treatment of UC.

Apart from the herein mentioned therapeutic agents, other inhibitor classes such as anti-topoisomerase I (Irinotecan) and histone deacetylase inhibitors (vorinostat) are used at the moment in clinical trials for the treatment of advanced and metastatic UC (ClinicalTrials.gov), but further investigations are needed to clarify their role in bladder cancer treatment.

Conclusion

Although bladder cancer is one of the leading tumors in western world, very little is still known about the molecular mechanisms that determine tumor formation in the bladder urothelium and the process of its metastatization. A better understanding of the molecular biology of bladder cancer will undoubtedly influence the selection of new therapeutic modalities. The value of integrating new biologically targeted agents into combined modality treatment for patients with metastatic bladder cancer has still to be proven. However, efficiently designed and rationalized trials, targeting therapeutic approaches to the molecular and histological characteristics of urothelial cancers, hold promise to improve the current results of metastatic bladder cancer treatment.

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