Histol Histopathol (2003) 18: 969-980

http://www.hh.um.es

Histology and Histopathology

Cellular and Molecular Biology

Review

Gene products involved in metastasis of bladder cancer

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Summary. Metastasis is usually responsible for mortality in patients suffering from muscle invasive bladder cancer. Whilst expression of a great number of genes and their protein products have been associated with metastasis and/or poor prognosis in bladder cancer, evidence that they actively drive the metastatic process, and hence make potentially good therapeutic targets, is often lacking. This is due to the limited number and application of effective animal models which reflect the pathogenesis of the human disease. In this review I will discuss the processes involved in metastasis, consider the established animal models of bladder cancer progression and metastasis, and review the evidence for a role of various gene products in this process. Consideration of clinical studies in conjunction with evidence from experimental animal models reveals that the tyrosine kinase receptor erbB1/EGFR, the calcium binding protein S100A4 and the the cell cycle arrest/apoptosis-inducing p53 protein are amongst the most promising targets for therapy against metastatic disease in patients with bladder cancer.

Key words: Bladder cancer, Metastasis, Gene products, Survival, Animal models

Clinical perspective

Carcinoma of the urinary bladder ranks as the fifth most common cancer in men and the eleventh in women in Western society. Transitional cell tumours of the bladder vary greatly in their capacity to invade adjacent stroma resulting in local progression, and to disseminate widely giving rise to distant metastases. At presentation, approximately 25-30% of bladder tumours are classified as muscle-infiltrative tumours which, by definition, have already demonstrated the ability to invade and are associated with a significant risk of metastasis (30-60%). Patients with these tumours have a significantly reduced 5-year survival rate (40%), often correlated to the development of metastases following the failure of conventional treatment strategies such as radical surgery or radiotherapy. Once a diagnosis of metastatic bladder cancer is made, the outlook is grave with a median survival of 12 months only for this group of patients.

Most would consider transitional cell carcinoma of the urothelium a chemosensitive tumour, and a large proportion of patients with advanced disease receive combination chemotherapy, usually with a cisplatin-based regimen. However, well over a decade since their introduction there is increasing awareness of the limitations of these aggressive regimens. The development of more specific, targetted therapies to treat metastatic disease is necessary to improve the survival of this group of patients. The objective of this review is to consider which known gene products are potentially good candidates for therapy in metastatic bladder cancer.

The metastatic cascade

Like tumourigenesis, the process of metastasis, in which cells escape from their tissue of origin and form secondary tumours at distant sites, is a multi-step phenomenon in which tumour cells must overcome a series of formidable barriers. Consequently, whilst at the level of the individual suffering from bladder cancer, metastasis is a relatively frequent occurrence, at the cellular level metastasis is a very infrequent and inefficient process. The metastatic cell must gain access to one of the circulatory systems, survive transit to the distant site, arrest, move out of the circulation, and establish a secondary tumour colony. It is unlikely that a single gene product can confer all of these abilities on a non-metastatic cell. Rather, it is probable that metastasis is a result of a stochastic process of mutation and deregulation of expression of multiple gene products. In searching for genes involved in metastasis, it is difficult to distinguish "metastasis-associated" genes from "metastasis-determining" genes. Metastasis-determining genes may act as master genes whose protein products control changes in the expression of other genes more intimately involved in various aspects of metastatic behaviour. It is hoped that deregulation of a limited number of metastasis-determining genes is rate limiting

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for the generation of the metastatic phenotype. The products of such genes are potential targets for therapy.

It is important to consider that in many muscle-invasive cancers, tumour cells may already have completed most of the stages of the metastatic cascade at the time of diagnosis, even if no secondary tumours are apparent. Quantitative studies using intravital videomicroscopy have shown that a major rate limiting step of the metastatic cascade in several experimental models of solid tumours, including breast cancer and melanoma, is the ability of single tumour cells at the secondary site to proliferate and form a clinically significant secondary tumour (MacDonald et al., 2002; Naumov et al., 2002). This suggests that tumour cells may readily enter the circulation and extravasate at distant sites, where they remain dormant for long periods. A greater understanding of the mechanisms that allow the tumour cell to "re-awaken" in a foreign tissue environment may be very important when designing therapeutic approaches. These mechanisms may involve an acquired ability to respond to local growth factors, autonomous growth, resistance to growth inhibitors, and promotion of angiogenesis.

Animal models

The vast majority of studies in the literature which have aimed to further our understanding of the molecular oncogenetics of bladder cancer metastasis have been correlative studies which, whilst suggesting potential therapeutic targets, do not distinguish between metastasis-associated and metastasis-determining genes. The development of reproducible, well defined animal models is essential in order to progress this field significantly. The most relevant model systems developed to date are those that involve orthotopic implantation of bladder cancer cells into the bladder wall or intravesical instillation into the bladder via a catheter. Orthotopic model systems have been developed in which rodent tumour cells are implanted into the bladders of immunocompetent animals, and human tumour cells are implanted into the bladders of nude mice. It has been reported that orthotopic models of bladder and other cancers are more likely to grow and metastasise from an orthotopic site (Ibrahiem et al., 1983; Theodorescu et al., 1990; Fu et al., 1991). Multiple sequential rounds of orthotopic implantation of a human bladder cancer cell line into the bladder wall, culture of tumour cells in vitro and re-implantation into the bladder wall of fresh hosts generates progressively more metastatic variants (Dinney et al., 1995). The recent development of transgenic mouse models of bladder cancer using urothelial-specific promotors such as the uroplakin II promoter (Lin et al., 1995) shows considerable promise. Expression of single oncogenes such as H-ras and EGF-R only causes hyperplasia and/or superficial tumour development (Zhang et al., 2001; Cheng et al., 2002) whilst the oncoprotein SV40 Large T antigen, which binds and inactivates a number of cell cycle proteins including p53 and Rb, causes carcinoma *in situ* and stochastic development of carcinoma (Zhang et al., 1999). Clearly, experiments that seek to investigate the mechanisms of metastasis in these model systems require the generation of more advanced models in which multiple oncogenes are expressed or tumour suppressor genes are mutated in a tissue-specific fashion. However, in another transgenic mouse model in which SV40 Large T antigen was expressed under the control of the cytokeratin 19 promotor, all transgenic mice in one lineage developed invasive bladder cancers, with intravascular lung metastases occurring in 20% of the affected mice (Grippo and Sandgren, 2000).

Metastasis-associated processes

Metastasis involves the cellular processes of invasion, migration (motility), growth and angiogenesis. The regulation of these processes is complex and can involve (1) inducers and suppressors produced by the tumour cells themselves, and (2) reciprocal interactions with normal cells. For example, angiogenesis is activated by a change in the balance of inhibitory and stimulatory polypeptide factors in favour of stimulation; these factors are produced by normal cells and the cancer cells, and can be stimulated by hypoxia. Similarly, the process of invasion is largely determined by an imbalance of proteolysis involving increased activity of proteases, which are produced as inactive precursors, and inhibitors of proteases; these factors may also be produced by normal cells. Metastasis also requires changes in adhesion, but these are complex and involve disruption of local cell-cell interactions on the one hand, and the promotion of inappropriate cell-cell interactions on the other e.g. adhesion of metastatic cells to organ-specific endothelial cells may, at least in part, explain organ specificity of metastasis. Another feature that metastasis shares with tumorigenesis is that it involves both dominantly acting genes which promote metastasis and repressor genes which suppress metastasis. I shall now discuss the roles of individual gene products in metastasis of bladder cancer.

Growth factors and their receptors

Although metastasis is a late event in the natural history of carcinogenesis, early gene changes can influence later events. Growth factors and their receptors are often perceived to be early changes due to their ability to act as oncogenes in some systems, and their ability to induce transition through the cell cycle and so induce proliferation. However, it has become apparent that signalling through growth factor receptors can have far more diverse effects on cells, including effects on motility through regulation of the cytoskeleton, protection from apoptosis, effects on cell shape, cellular adhesion, and expression of a variety of genes unrelated to cell growth such as proteins that modulate invasion.

Tyrosine kinase receptors of the erbB family have

been strongly implicated in progression of bladder cancer to the metastatic phenotype. This family of growth factor receptors consists of four members which participate in a network of ligand-receptor interactions. The ligands, which include EGF, TGFα, heregulin, neuregulin and heparin-binding EGF, bind to one receptor with a high affinity and narrow specificity, and another with low affinity but wide specificity. Ligands can signal through heterodimers or homodimers of erbB receptors. Although erbB2 is the preferred low affinity receptor for the various ligands, the preferred high affinity receptor varies for each ligand, and may be erbB1, erbB3 or erbB4. The strength of the transduced intracellular signal depends on the ligand-receptor dimer combination. Both erbB1 (EGF receptor) and erbB2 have been shown to be overexpressed in muscle invasive bladder cancer (Neal et al., 1986; Lipponen, 1993). Expression of erbB1 has been shown to be a significant independent prognostic indicator of poor survival (Mellon et al., 1995) whereas although erbB2 expression did not predict survival, overexpression in the primary tumours consistently predicted overexpression in distant and regional metastases (Jimenez et al., 2001). The expression of EGF-R in a panel of 20 metastatic lesions reflected the pattern of expression in the primary bladder tumours from which they were derived (Bue et al., 1998).

There is strong evidence from animal models that EGF-R/erbB1 plays a deterministic role in bladder cancer metastasis. The EGF-R inhibitor 4,5-dianilinophthalimide (CGP 54211) has been shown to inhibit orthotopic growth of the highly metastatic human TCC line 253J B-V in nude mice (Dinney et al., 1997) and the anti-EGF-R antibody C225 can inhibit angiogenesis of this same cell line in vivo (Perrotte et al., 1999). The inhibition of angiogenesis was accompanied by down-regulation of the mRNA and protein expression of the angiogenic factors VEGF, IL-8 and bFGF (Perrotte et al., 1999). The reduction of tumorigenicity by C225 but not the incidence of metastasis was enhanced by paclitaxel; this agent also enhanced apoptosis in tumour and endothelial cells compared with C225 alone (Inoue et al., 2000a).

Another reason why EGF-R is a very strong candidate for a metastasis-determining gene in bladder cancer is because it has been shown to modulate multiple phenotypes associated with metastasis; in addition to angiogenesis, these include invasion and motility. The molecular mechanisms underlying these associations are beginning to be elucidated. For example, it is known that activation of the AP-1 transcription factor, which occurs following EGF-R stimulation, can induce expression of certain collagenases including MMP-1 and 9, that have been implicated in invasion. EGF stimulation of human bladder carcinoma cells increases MMP-1 mRNA levels markedly (whereas MMP-2 mRNA remained constant) and elevated levels of MMP-1 are detected in the urine of patients with high stage bladder tumours (Nutt et al., 1998). It has also been reported that EGF stimulation of KU-I human bladder carcinoma cells lead to enhanced anchorage-independent cell growth and increased numbers of cells penetrating a matrigel membrane (Kanno et al., 1998). In this cellular system, EGF stimulation also altered the cancer cell morphology and increased promoter activity for MMPs 1 and 9. EGF has been shown to stimulate motility in bladder tumour cell lines harbouring H-ras mutations via the small GTP binding proteins RalA and RhoA but independent of Rac1 and cdc42 (Gildea et al., 2002).

Other growth factors that have been implicated in metastasis of bladder cancer include Hepatocyte Growth Factor (HGF), Platelet-Derived Endothelial Cell Growth Factor (PD-ECGF), Vascular Endothelial Growth Factor (VEGF), and basic Fibroblast Growth Factor (bFGF or FGF-2). Levels of HGF in serum and tissues of bladder cancer patients are significantly greater in patients with muscle-invasive disease than those with superficial disease, with the highest levels of elevation in patients with visceral metastasis. Moreover, the serum level of HGF is significantly inversely correlated with disease-free and overall survival rates (Gohji et al., 2000). However, although transfection of HGF has been shown to promote motility and scattering of NBT-II mouse bladder cancer cells in vitro, and to induce more rapidly growing tumours in vivo, no induction of metastasis was reported (Bellusci et al., 1994). In rat bladder cancer cells, HGF stimulated migration, invasion and secretion of MMPs. Moreover, HGF mRNA and protein expression were shown to be induced in rat bladder stromal cells by medium conditioned by rat bladder cancer cells (Tamatani et al., 1999). A significant correlation has also been found between expression of PD-ECGF, a factor shown to stimulate endothelial cell chemotaxis, and histologic grade, infiltration, local invasion and lymph node metastasis in bladder cancer. Moreover, in multivariate analysis, expression of this factor was found to be an independent indicator of poor prognosis (Arima et al., 2000).

Vascular Endothelial Growth Factor (VEGF) has been shown to trigger the angiogenic switch in a wide variety of tumour types. Elevated serum levels of VEGF is significantly associated with high grade and stage, vascular invasion and carcinoma in situ, and presence of metastasis. However, although a serum level of VEGF in excess of 400 pg/ml was significantly related to reduced disease-free survival, it did not remain an independent prognostic indicator on multivariate analysis (Bernardini et al., 2001). The anti-VEGF monoclonal antibody DC101, in combination with paclitaxel, significantly inhibited spontaneous metastasis and neovascularisation of 253J B-V cell tumours growing orthotopically in the bladders of nude mice, compared with either agent alone. In this system, the inhibition of angiogenesis was accompanied by enhancement of tumour and endothelial cell apoptosis (Inoue et al., 2000b).

The FGF family of growth factors are also well known angiogenic factors and may also play a role in the

metastasis of bladder cancer, although only FGF-2 has been studied in any detail to date. Expression of FGF-2 has been shown to regulate production of MMPs 2 and 9 and invasive potential in human bladder cancer cell lines in vitro (Miyake et al., 1997). Treatment of highly metastastatic 253J B-V cells with Interferon α down-regulated production of FGF-2, and decreased blood vessel density when these cells were grown as tumours in the bladder wall of nude mice (Dinney et al., 1998). Antisense to FGF-2 inhibited expression of MMP-9 in 253J-BVR cells growing subcutaneously as tumours in nude mice, whilst concurrently inhibiting proliferation and enhancing apoptosis of endothelial cells in these tumours (Inoue et al., 2000c). In the NET-II rat model of bladder cancer, only transfection of the 24KDa form of FGF-2, which localises to the nucleus, resulted in spontaneous lung metastases from a subcutaneous site and experimental lung metastases when injected intravenously; transfection of the 18 kDa cytoplasmic isoform failed to induce metastasis from either site. These experiments suggested that FGF-2 can induce metastasis in bladder cancer via nuclear targets, and independently from an FGF-receptor mediated pathway (Okada-Ban et al., 1999). However, tumour cell immunoreactivity for FGF-2 in the majority of human bladder cancers has been reported to be weak, with intense immunoreactivity being more common in blood vessels within the tumours, normal detrusor muscle and the basal lamina of normal transitional epithelium, suggesting that the major source of FGF-2 in bladder cancer may be degradation of epithelial basement membranes and detrusor muscle (O'Brien et al., 1997).

Whilst growth factors such as VEGF and FGF-2 are potent inducers of angiogenesis, the ability of a tumour to vascularise can also be influenced by other proteins including angiogenin, and ECM components such as thrombospondin. Bladder cancer patients with elevated levels of serum angiogenin were found to have significantly lower overall survival. Moreover, in patients with advanced bladder cancer who underwent complete resection, their disease-free survival was highly significantly worse when their serum level of angiogenin was elevated (Miyake et al., 1999). Expression of thrombospondin has also been inversely correlated with survival and microvessel density in bladder cancer (Grossfeld et al., 1997).

Proteolysis

It has already been discussed above that signalling through growth factor receptors such as EGF-R can result in induction of transcription of certain members of the matrix metalloproteinase (MMP) family. It is well established that metastatic tumours have an imbalance of proteolysis favouring invasion. Extracellular protein degradation is controlled by proteolytic enzymes, among the important of which are endopeptidases which are secreted as inactive precursors or proenzymes, and must be activated by other proteases. Tissue inhibitors of activation also exist, such as Tissue Inhibitors of Metalloproteinases (TIMPs), which are broad-spectrum inhibitors that bind to and inactive MMPs. Hence, invasion can be influenced by either the amounts of proenzyme, activating proteases or inhibitors. Two major types of protease are secreted by cancers, categorised according to whether or not they require zinc or calcium ions; these the the metalloproteinases (MMPs); collagenase types I and IV and stromolysins require the metal ions, whereas those that do not require the metal ions have a serine residue, such as plasmin. Plasminogen is the inactive precursor of plasmin; this serine protease is an important activating protease which can convert procollagenases and prostromolysins to the corresponding active enzymes. The activation of plasminogen is regulated by another serine protease, called urokinase type plasminogen activator (uPA). Deregulation of most of these proetins has been implicated in bladder cancer metastasis.

The levels of MMPs 2 and 9, as measured by zymography, showed significant correlations with the grade and invasiveness of bladder cancer, but the genes were expressed chiefly in the stroma rather than the epithelial tumour cells (Davies et al., 1993). In contrast, Grignon et al. reported that immunoreactivity for MMPs 2 and 9 was predominantly tumour cell-associated, whereas TIMP2 was predominatly detected in the stroma. These authors paradoxically reported that strong staining for TIMP2 was associated with poor patient prognosis, whilst MMP2 and MMP9 expression did not correlate with stage, grade or outcome (Grignon et al., 1996). However, other studies have shown that TIMP2 expression (Kanayama et al., 1998) and MMP2 expression (Kanayama et al., 1998; Kanda et al., 2000; Nakanishi et al., 2000) are associated with decreased survival in patients with bladder cancer. The presence of urinary MMP1 has been associated with muscle invasive disease, stage progression and poor survival, whereas high urinary TIMP1 levels were associated with muscle-invasive disease and progression but not disease-specific survival (Durkan et al., 2001). The findings that expression levels of TIMPs are at variance with their roles as inhibitors of MMPs has been sunstantiated by a recent report that the expression level of TIMP1 mRNA correlates with Ki-67 labelling index and poor prognosis in bladder cancer (Yano et al., 2002). Bianco et al. have reported that MMP9 expression in bladder washes from bladder cancer patients predicts pathological stage and grade (Bianco et al., 1998).

There is little functional data on roles of MMPs and TIMPs in tissue culture and animal models of bladder cancer. Transfection of invasive but non-metastatic rat MYU-3L bladder cancer cells with human pro-collagenase type IV induced the metastatic phenotype in these cells (Kawamata et al., 1995), whilst transfection of the metastatic rat bladder cancer cell line LMC-19 with TIMP1 or TIMP2 significantly inhibited the extravascular growth of pulmonery eboli (Kawamata et al., 1995). The latter result suggested that MMPs may be

critical inducers of extravasation at this site of metastasis. This is interesting given the apparently paradoxical positive correlation between expression of TIMPs in primary tumours of human patients with bladder cancer and prognosis. Invasion of the human bladder cancer cell line T24 was increased by the tumour promotor TPA and decreased by N-acetylcysteine; this was correlated with a modulation of expression and activity of MMP-9 (Kawakami et al., 2001). Finally, the alkaloid halofuginone has been shown to inhibit activity of the MMP-2 promotor and down-regulate expression of the MMP-2 gene in murine (MBT2-t50) and human (5637) bladder cancer cells. Halofuginone-treated cells failed to invade through matrigel, and caused a marked reduction in lung colonisation of MBT2 cells after i.v. inoculation (Elkin et al., 1999).

Immunoreactivity for Urokinase-type plasminogen activatior and its inhibitor has been reported to be of no value in determining the prognosis of patients with upper urinary tract TCC (Nakanishi et al., 1998). However, another protease, heparanase, has been shown to be elevated in muscle invasive and lymph node metastatic bladder cancer relative to superficial and lymph node negative bladder cancer respectively (Gohji et al., 2001a,b). Moreover, expression of this protease was correlated with high microvessel densities and significantly lower patient survival (Gohji et al., 2001b).

Cytokines and chemokines

Interferons α and ß have been shown to modulate the metastatic potential of experimental animal models of human bladder cancer. Treatment of IFN-α resistant highly metastatic 253J B-V-R cells with IFN-α down-regulated MMP-9, up-regulated E cadherin and inhibited invasion *in vitro*, whilst systemic therapy with IFN-α decreased expression of MMP-9, increased expression of E-cadherin and inhibited metastasis. This study showed that IFN-α can limit tumour invasion by restoring the normal balance between MMP-9 and E-cadherin (Slaton et al., 2001). Expression of the Interferon-ß (IFN-ß) gene in metastatic 253J B-V-R cells significantly inhibited both tumorigenicity and lymph node metastasis, and induced necrosis and sequestration of activated macrophages within the tumours. Moreover, expression of FGF-2 and MMP-9, two other gene products associated with bladder cancer metastasis, were down-regulated in this model system by IFN-ß (Izawa et al., 2002).

Other cytokines implicated in bladder cancer metastasis include IL-8 and IL-6. IL-8 has been shown to up-regulate MMP-9, collagenase activity and invasive potential in non-metastatic 253J-P cells *in vitro*, and to increase microvessel density and induce spontaneous metastasis when this cell line was implanted into the bladder wall of nude mice (Inoue et al., 2000d). Elevated levels of IL-6 and its soluble receptor are associated with invasion and lymph node metastasis in human bladder cancer, where they have also been shown to be independent predictors of these phenotypes and disease-specific survival in multivariate analyses (Andrews et al., 2002).

The potential relationship between production of chemokines and metastasis in bladder cancer has received very little research attention. The level of the chemokine monocyte chemo-attractant protein 1 (MCP-1), a potent monocyte chemoattractant to tumour sites, in urine correlates significantly with tumour stage and the presence of metastasis (Amann et al., 1998). A monocyte/macrophage infiltrate may facilitate tumour neovascularisation and invasion.

Adhesion molecules

Cell adhesion molecules mediate interactions between cells and their immediate environment. Three types of adhesive process are important: (1) recognition of similar cells (homotypic adhesion), (2) recognition of dissimilar cells (heterotypic adhesion), and (3) cell-ECM interactions. The major homotypic adhesion molecule which mediates interactions between urothelial cells is E-cadherin. There is considerable evidence that down-regulation of E-cadherin is an important step in bladder cancer metastasis. Loss of membranous E-cadherin immunoreactivity has been correlated with advanced tumour stage, invasion and presence of metastasis in human bladder cancer (Syrigos et al., 1995). Abnormal E-cadherin immunoreactivity correlated strongly with progression and poor outcome (Popov et al., 2000; Byrne et al., 2001), and along with stage, appears to have significant additional prognostic value (Popov et al., 2000). Moreover, study of E-cadherin mRNA and protein expression in the same group of patients has shown that down-regulation of E-cadherin expression in bladder cancer appears to occur at the transcriptional level (Popov et al., 2000).

Loss of normal cell surface expression of p120, a protein which forms complexes between E-cadherin and the cytoskeleton, has also been correlated with poor survival in bladder cancer (Syrigos et al., 1998). Abnormal expression of the catenins, another group of proteins that link E-cadherin to the actin cytoskeleton, have also been implicated in bladder cancer metastasis. Down-regulation of ß catenin has been correlated with presence of distant metastasis whilst abnormal expression of α catenin was associated with poor survival (Mialhe et al., 1998). Finally, it has been shown that lymphocyte function-associated antigen-1 (LFA-1) can enhance the adhesion between ICAM-1 expressing RT4 bladder cancer cells and human umbilical vein endothelial cells, suggesting that LFA-1-expressing leukocytes may act as a bridge between the endothelium and ICAM-1-expressing tumour cells (Tanabe et al., 1997). This may potentially enhance arrest of such tumour cells at a secondary site.

p53, the cell cycle, and apoptosis-associated genes

The p53 gene is the most commonly mutated gene in human cancer, and plays a crucial role in protecting the

genome from the accumulation of genetic damage. In response to DNA damage and various other types of cellular stress, p53 is stabilised and activates expression of a number of target genes, including the cyclin-dependent kinase inhibitor p21, which arrest the cell cycle via a mechanism which involves the retinoblastoma (Rb) protein. Under certain circumstances, activation of p53 can cause induction of apoptosis, via the induction of expression of pro-apoptotic genes including bax. Unless activated, wild-type p53 has a short half life, and detection of its expression in cancers is indicative of an inactivating mutation in the gene, which results in post-translational stabilisation of the protein. Immunostaining for p53 has been associated with tumour stage, grade, and metastasis in bladder cancer (Moch et al., 1993). p53 immunostaining has been reported to be an independent prognostic factor for superficial TCC (Serth et al., 1995). In a large multivariate analysis of 177 variables in 210 patients with T1 and T2a bladder cancer, expression of p53 was found to be the sole variable to provide an independent prediction of metastasis (Rodriguez-Alonso et al., 2002). However, some studies have failed to show a relationship between p53 staining and survival in bladder cancer (Siu et al., 1998; Tiguert et al., 2001).

The relationship between p53 status and response to therapy has also been studied in patients with bladder cancer. In a study of primary bladder tumours from 50 pateints who developed metastatic bladder cancer, the response to platinum-based combination chemotherapy and survival of the patients was not dependent on p53 nuclear reactivity (Sengelov et al., 1997). In another study of patients treated with pre-operative radiotherapy, staining for p53 was only of prognostic value in T3b patients; in this subgroup p53 staining was associated with a higher rate of distant metastasis (Wu et al., 1996). Also, p53 staining has been reported not to be predictive of disease-free survival in patients with node-positive disease (Fleshner et al., 2000). Raitanen et al. have reported that patients with metastatic bladder cancer tend to be non-diploid and positive for p53, with further genetic changes being uncommon, whilst superficial recurrences are mostly diploid and negative for p53 (Raitanen et al., 1997). Moreover, in a study of 31 T2 and T3a tumours, 17 (55%) were found to be positive for p53. Eleven of these 31 patients had lymph node metastases, all of which had primary tumours which stained for p53 (Uygur et al., 1999). An association has been reported between p53 status and degree of angiogenesis (Bochner et al., 1997). Other cell-cycle associated proteins that function in the G1-S transition or G1-S checkpoint have also been implicated in progression of bladder cancer. Loss of p21 expression has been shown to be an independent predictor of progression in bladder cancer (Stein et al., 1998). Moreover, low nuclear reactivity for E2F-1, a transcription factor that binds to the retinoblastoma protein pRb, has been significantly associated with progression to metastasis and death in bladder cancer patients (Rabbani et al., 1999). There is support for an important role for p53 in bladder cancer metastasis from animal models. A full length antisense p53 construct was sufficient to convert an immortalised but non-tumourigenic rat urothelial cell line to a muscle-invasive carcinoma cell line which metastasised to lungs in 3/7 orthotopically-injected nude mice (Okamoto et al., 1998). The finding of lung metastases in cytokeratin 19-SV40 Large T antigen transgenic mice (Grippo and Sandgren, 2000) also supports a role for either p53 or one of the other cell cycle-associated proteins which bind to this oncoprotein in metastasis of bladder cancer. The generation of urothelium-specific knockouts of these individual genes will undoubtedly shed more light on their roles in progression of bladder cancer.

An animal model system has also suggested a role for the anti-apoptotic bcl-2 gene in bladder cancer metastasis. Over-expression of bcl-2 in KoTCC-1/BH human bladder cancer cells enhances lung colony formation after intravenous injection and spontaneous metastasis to lymph nodes following injection into the bladder wall of mice. These cells also exhibited anti-apoptotic activity under anchorage-independent conditions in vitro (Miyake et al., 1999). In clinical studies, no correlation has been found between expression of bcl-2 and progression or prognosis of human bladder cancer (Shiina et al., 1996; Glick et al., 1996), or distant metastasis (Pollack et al., 1997a,b). However, overexpression of bcl-2 has been suggested to play a role in thwarting the apoptotic response to radiation in muscle-invasive bladder cancer (Pollack et al., 1997a,b).

Cox-2

Cyclooxygenase (Cox)-2, an enzyme involved in prostaglandin synthesis, has also been strongly implicated as an angiogenic agent. Cox-2 expression has been demonstrated in invasive and non-invasive bladder tumours, but high expression of this enzyme is associated with invading cells (Ristimaki et al., 2001). Indeed, Cox-2 expression has been shown to be associated with local, lymphatic and venous invasion. However, Cox-2 expression has not been shown to be an independent prognostic indicator of survival (Shirahama et al., 2001).

Other proteins

A number of other proteins have been associated with bladder cancer metastasis, which either do not fit into any of the categories above, or their function is poorly understood. These include S100A4, BRMS1, RhoGDI2, nm23, KAI1 (CD82), Glut1, clusterin and pS2.

S100A4 is a small calcium-binding protein containing EF-hand motifs which associates with the cytoskeleton. In the mouse, it was first identified as a gene differentially expressed in metastatic versus non-

metastatic mouse mammary epithelial cell lines. Its expression is associated with stage progression, metastasis and poor survival in human bladder cancer (Davies et al., 2002). Moreover, transfection of S100A4 into the invasive but non-metastatic rat bladder cancer cell line MYU-3L generates metastatic variants (Levett et al., 2002).

BRMS1 and RhoGDI2 have been identified as potential metastasis-associated genes by characterising the human bladder cancer cell line T24 and a more metastatic lineage related variant of it, termed T24T. In this model system, BRMS1 mRNA was expressed at a higher level in the less aggressive variant, whereas RhoGDI2 was expressed exclusively in the less aggressive variant (Seraj et al., 2001).

Nm23, which was first identified as a metastatsis-suppressor gene in melanoma and subsequently in other model systems, including breast cancer, has been reported to be more highly expressed in highly proliferative and invasive TCCs than in superficial

Table 1. Comparison between evidence from clinical studies and animal model systems for the role of various gene products in metastasis of bladder cancer

GENE PRODUCT	RELATIONSHIP WITH METASTASIS/ PROGNOSTIC VALUE IN HUMAN BLADDER CANCER	EVIDENCE FOR ROLE IN METASTASIS IN ANIMAL MODELS
erbB1 (EGFR)	Metastasis/significant independent prognostic indicator of survival.	Growth, angiogenesis, invasion, motility and metastasis of human bladder cancer cell line in orthotopic model. Increases MMP production
erbB2	No relationship with survival	None
HGF	Serum level correlates with survival	Promotes scattering and motility of MBT-2 cells in vitro but no effect on metastasis of these cells in vivo
PDGF	Metastasis/significant independent prognostic Indicator of survival	None
FGF-2	None reported	24KDa form induces spontaneous metastases in orthotopic model
Angiogenin	Elevated serum level associated with poor survival.	None
Thrombospondin	Inversely correlated with survival	None
TIMP1	No association with survival	Inhibits growth of pulmonary emboli in rat model
TIMP2	Associated with poor survival	Inhibits growth of pulmonary emboli in rat model
MMP1	Associated with poor survival	None
MMP2	Controversial	None
MMP9	No association with survival	None
Heparanase	Associated with poor survival	None
IFNα	None reported	Systemic administration to mice inhibits metastasis of orthotopically growing human bladder cancer cells. Increases MMP9, and down-regulates E cadherin
IFNßß	None reported	Transfection into human bladder cancer cell line inhibits tumourigenicity and metastasis from orthotopic site, induces necrosis and sequesters macrophages
IL-8	None reported	Transfection into human bladder cancer cell line induces spontaneous metastasis and up-regulates MMP9
IL-6	Independent prognostic indicator of poor survival.	None
E Cadherin	Metastasis/poor survival	None
p120	Poor survival	None
α catenin	Poor survival	None
ß catenin	Metastasis	None
p53	Metastasis/relationship with prognosis controversial.	Antisense reduces metastasis in orthotopic rat model. Lung metastases develop from bladder tumours in SV40 Large T antigen transgenic mice.
E2F-1	Low expression associated with poor survival	None
Bcl-2	No relationship reported	Overexpression in human bladder cancer cell line results in metastasis from orthotopic site and anti-apoptotic activity
S100A4	Metastasis/poor survival	Induces spontaneous metastasis from orthotopic site in rat model
Nm23	Controversial	None
Clusterin	None reported	Antisense Oligonucleotides inhibit growth and metastasis
pS2	Poor survival	None

bladder cancers or normal urothelium (Siina et al., 1995), but was not associated with the early development of metastases or a favourable clinical outcome (Shiina et al., 1995; Alderisio et al., 1998). However, a more recent study reported an inverse correlation between nm23 expression and the occurrence of metastasis and poor survival in grade 2 bladder tumours (Chow et al., 2000).

KAI1, located on chromosome 11p11.1, encodes a transmembrane glycoprotein and has been identified as a metastasis suppressor gene in human prostate cancer. KAI1 mRNA and protein expression significantly decrease in invasive bladder cancers compared with non-invasive cancers (Yu et al., 1997; Ow et al., 2000). Low KAI1 mRNA levels also correlated with increased in vitro invasive ability, along with reduced cell-cell and cell-ECM adhesion (Jackson et al., 2000). It has been reported that p53 can activate the KAI1 gene by interacting with the 5' upstream region (Mashimo et al., 1998). However, whilst a correlation between KAI1 and p53 expression has been shown in prostate cancer (Mashimo et al., 1998), no such correlation was found in bladder cancer (Ow et al., 2000; Jackson et al., 2002).

Down-regulation of the clusterin gene by antisense oligonucleotides has been shown to inhibit in vivo growth and metastasis, and to enhance cisplatin-induced apoptosis in s.c. or orthotopically implanted KoTCC-1 cells (Miyake et al., 2001). Expression of the glucose transporter Glut1 has been shown to be associated with poor patient survival but not incidence of lymph node metastasis (Younes et al., 2001). The expression two poorly characterised glycoproteins, metanestin and Sialosyl-Le(x), have also been associated with metastasis of bladder cancer (Takemoto et al., 1997; Numahata et al., 2002). Expression of the oestrogen-regulated pS2 protein is switched on in approximately 40% of human bladder tumours and is associated with poor survival. Moreover, the fraction of positive cells was higher in invasive tumours with lymph node metastasis (Lipponen and Eskelinen 1994).

Synthesis, conclusions and future perspectives

In this review I have considered the evidence for the involvement of known gene products in the process of metastasis of bladder cancer in both clinical studies of human bladder cancer and functional studies in animal models. The data for each gene product is summarised in Table 1. It is clear that some gene products, such as bcl-2 and FGF-2, have been implicated in bladder cancer metastasis in animal model systems but not in the human disease. Conversely, others, such as HGF, E-cadherin and E2F-1 are associated with a poor prognosis in patients with bladder cancer, but evidence that they drive the metastatic process from animal models is lacking. In the case of TIMP-2 the data appears to be conflicting with an association with poor survival being reported in the human disease even though it has been shown to inhibit the growth of pulmonary emboli in a rodent model. In order to resolve these issues and extend our knowledge of the process of metastasis in bladder cancer, the range, relevance and application of animal models needs to be extended. Taking the clinical and animal model data together, the most promising candidates for therapy against metastatic disease in bladder cancer at present appear to be erbB1/EGFR, S100A4 and possibly p53. It is also highly likely that one or more of the adhesion molecules may play a deterministic role in the metastatic process but the functional roles of these proteins have not been studied in animal models of bladder cancer.

It remains to be determined whether transfection of EGF-R into non-metastatic bladder cancer cells can induce the metastatic phenotype. Moreover, the potential therapeutic benefit of EGF-R-specific inhibitors is under evaluation in animal models of bladder cancer. S100A4 protein may also prove to be a suitable target for therapy, but at the present time its function is poorly understood. Moreover, whilst a number of proteins have been shown to bind to S100A4, including p53 and various cytoskeletal proteins, the impact of these interactions on the metastatic process is not known. The role of p53 (and other potential metastasis-suppressor genes) in bladder cancer metastasis will be more fully understood once this gene can be specifically inactivated in bladder cancer, perhaps by breeding the established transgenic mice over-expressing H-ras or EGF-R in the urothelium with loxP flanked p53, which can then be removed by delivering cre recombinase somatically into the bladder lumen or by creating uroplakin-cre transgenic mice. Application of genomic and proteomic technologies will undoubtedly uncover new candidate genes involved in bladder cancer metastasis, but assessing their significance will depend, as with the current candidate genes, on the application of appropriate animal model systems relevant to the human disease.

Acknowledgements. I thank my clinical colleagues past and present, particularly David Neal, Kilian Mellon and John Kelly, members of my research group, and Cancer Research UK for ongoing financial support. I also gratefully acknowledge John Kirby for critically reading the manuscript.

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Accepted February 26, 2003