REVIEW

Molecular subtypes of bladder cancer: Jekyll and Hyde or chalk and cheese?

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Cancer of the bladder shows divergent clinical behaviour following diagnosis and it has been proposed that two major groups of tumours exist that develop via different molecular pathways. Low-grade, non-invasive papillary tumours recur frequently, but patients with these tumours do not often suffer progression of disease to muscle invasion. In contrast, tumours that are invading muscle at diagnosis are aggressive and associated with significant mortality. Molecular studies have identified distinct genetic, epigenetic and expression changes in these groups. However, it is not yet clear whether there is direct progression of low-grade superficial tumours to become invasive (a Jekyll and Hyde scenario) or whether in those patients who apparently progress from one form of the disease to the other, different tumour clones are involved and that the two tumour groups are mutually exclusive (‘chalk and cheese’). If the latter is true, then attempts to identify molecular markers to predict progression of low-grade superficial bladder tumours may be fruitless. Similarly, it is not clear whether other subgroups of tumours exist that arise via different molecular pathways. There is now a large amount of molecular information about bladder cancer that facilitates examination of these possibilities. Some recent studies provide evidence for the existence of at least one further group of tumours, high-grade superficial papillary tumours, which may develop via a distinct molecular pathway. Patients with such tumours do show increased risk of disease progression and for these there may exist a real progression continuum from non-invasive to invasive. If this is the case, definition of the molecular signature of this pathway and improved understanding of the biological consequences of the events involved will be pivotal in disease management.

Introduction

Bladder cancer is a common disease affecting >12,500 patients in the UK and an estimated 63,000 in the US each year with 5000 and 13,000 deaths, respectively (1,2). Worldwide it is estimated that >300,000 new cases occur each year, with the highest incidence in industrialized countries and areas where infection with the parasite Schistosoma haematobium is endemic. In the latter case, many of the tumours that develop are squamous cell carcinomas. In contrast, the majority of non-schistosomiasis-related cases are transitional cell carcinoma and only this group of tumours will be discussed here. In the western world, bladder cancer is a disease of middle and old age. Smoking is a significant risk factor as are certain occupational carcinogens (3). There is a male:female ratio of ~3:1 and in men in the UK, bladder cancer is now the fourth most common malignancy.

A striking feature of urothelial cell carcinoma (UCC) is the existence of two distinct groups of tumours with different clinical features. More than 70% of tumours at diagnosis are non-invasive papillary lesions. These commonly recur but progression to muscle invasion is relatively infrequent (10–20%) and prognosis is good. In contrast, the ~20% of tumours that are muscle invasive at diagnosis, have a poor prognosis with <50% survival at 5 years. As discussed below, these groups are also distinct at the molecular level.

Although there is now extensive information on bladder cancer genetics, epigenetics and gene expression, several questions remain unanswered. A key issue is whether existing molecular information can explain disease pathogenesis in the two major tumour groups in terms of the preferred combinations and order of molecular events and of the biochemical signalling pathways involved. Similarly, although the identification of markers that will predict which superficial bladder tumours will later progress to become invasive has often been stated as a goal of molecular studies, to date molecular information has failed to provide robust predictive markers. Possibly, key markers still remain to be identified. Alternatively, superficial tumours never evolve to become invasive and these represent truly distinct and mutually exclusive entities. Possibly, these tumour groups are more analogous then to ‘chalk and cheese’ than to the Jekyll and Hyde, that they have been assumed to be. Or perhaps there are more than two major groupings that might be defined in molecular terms. This review will discuss these issues in the context of the current state of knowledge of the molecular biology of human bladder cancer. Much information has come from studies of carcinogen-induced tumours in rodents and from transgenic models in which some of the events commonly found in human bladder tumours have been induced in the mouse urothelium. These studies will not be discussed here but the reader is referred to an excellent review by Wu (4) for a more general discussion of bladder cancer including emerging therapeutic strategies that exploit molecular information and information from recent studies using transgenic mice.

Two major groups of bladder tumours: or more?

It has long been recognized that bladder tumours show divergent clinical behaviour that is associated with their histopathology at diagnosis. The majority of bladder tumours (70–80%)...
are superficial, exophytic, papillary tumours that are well-differentiated (low-grade) and do not penetrate the epithelial basement membrane. Such tumours are described as stage Ta (Figure 1a) (5). Histopathological observation suggests that these arise from the normal urothelium (Figure 1b) via hyperplasia and the development of a branching vasculature (Figure 1d). Such tumours are not usually associated with the high-grade lesion carcinoma in situ (CIS) (Figure 1c), which though strictly ‘superficial’ is composed of poorly differentiated (high-grade) epithelium and is considered a high risk lesion (5). Superficial papillary UCC recurs frequently and is often multifocal, but progression to muscle invasion is not common and it is debatable whether in patients in which progression occurs, it is the initial papillary tumour that progresses. For patients with this type of disease, monitoring for tumour recurrence is very important and currently this is achieved by regular cystoscopic observation with relatively high morbidity and significant costs. Such patients would greatly benefit from non-invasive (e.g. urine-based) methods for disease monitoring and improved post-surgical intravesical therapies.

Muscle invasive tumours are usually diagnosed in patients with no previous history of papillary tumours. These may penetrate muscle (T2) and extend beyond the bladder wall (T3) or into adjacent structures (T4) (Figure 1a). Commonly the tumour epithelium is poorly differentiated (high-grade) (Figure 1e) and such tumours are often associated with CIS elsewhere in the bladder, which is believed to represent the precursor lesion. Tumours that have penetrated the basement membrane but are not invading muscle (T1) have in the past been classified as ‘superficial’. Owing to the relatively worse prognosis, particularly for high-grade T1 tumours and the finding of many genetic alterations in common with ≥T2 tumours (see below), these are currently considered with the invasive tumours (5). For patients with invasive bladder cancer, metastasis is a major clinical problem and novel systemic therapies are urgently needed for this poor prognosis group.

This description fits the two extremes of the tumour spectrum very well. However, Ta papillary tumours of high-grade exist and these are often accompanied by dysplasia elsewhere in the bladder. Such tumours have a higher recurrence rate and patients with such tumours show increased risk of invasion. Possibly therefore, as has been suggested by Lee and Droller (6), higher grade papillary UCC may develop in a distinct pathway via urothelial atypia and dysplasia as in the case of the development of CIS, but accompanied by hyperplasia and ultimately the development of a papillary architecture.

Molecular alterations

Many studies have assessed the status of known oncogenes and tumour suppressor genes and have searched for common chromosomal alterations to identify novel genomic events in bladder cancer (7,8). Genetic and epigenetic changes involving known genes are summarized in Table I. Most studies

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**Fig. 1.** (a) Staging of UCC. (b–e) Haematoxylin and eosin stained sections. (b) Normal urothelium. Note the large differentiated superficial cells. (c) CIS. Cells are disorganized and show marked nuclear atypia (high-grade). (d) Low-grade superficial papillary tumour. (e) High-grade tumour invading muscle (m).
Molecular subtypes of bladder cancer

Table I. Genetic and epigenetic alterations of known genes in transitional cell carcinoma

<table>
<thead>
<tr>
<th>Gene (cytogenetic location)</th>
<th>Alteration</th>
<th>Frequency/clinical association</th>
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<tbody>
<tr>
<td>Oncogenes</td>
<td></td>
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<tr>
<td>HRAS (1p15)/NRAS (1p13)/KRAS2 (12p12)</td>
<td>Activating mutations</td>
<td>10–15% all grades and stages (9–12)</td>
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<tr>
<td>FGFR3 (4p16)</td>
<td>Activating mutations</td>
<td>30–80% (13,14) predominantly low-grade/stage</td>
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<tr>
<td>ERBB2 (17q12)</td>
<td>Amplification/overexpression</td>
<td>Amplified in 10–14% high-grade/stage (15–17)</td>
</tr>
<tr>
<td>CCND1 (11q13)</td>
<td>Amplification/overexpression</td>
<td>10–20% all grades and stages (18,19)</td>
</tr>
<tr>
<td>MDM2 (12q13)</td>
<td>Amplification/overexpression</td>
<td>4% amplification, high-grade; ~30% overexpression, low-grade (20,21)</td>
</tr>
<tr>
<td>E2F3 (6p22)</td>
<td>Amplification/overexpression</td>
<td>Amplification in tumour cell lines (22–24) and 11% ≥T1 tumours (25)</td>
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<tr>
<td>Tumour suppressor genes</td>
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<tr>
<td>RASSF1A (3p21)</td>
<td>Methylation</td>
<td>62% overall (26); high stage (27); progression (28)</td>
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<tr>
<td>FHIT (3p14)</td>
<td>Deletion/methylation</td>
<td>Deletion during urothelial transformation in vitro (29); LOH 44% overall, most in muscle invasive (30); 61% reduced expression (31); 16% methylation (32)</td>
</tr>
<tr>
<td>CDKN2A (9p21)</td>
<td>HD/methylation/mutation</td>
<td>20–30% high-grade/stage (33–35); LOH 60% all grades/stages; immunization in vitro (36)</td>
</tr>
<tr>
<td>PTCH (9q22)</td>
<td>Deletion/mutation</td>
<td>LOH 60% all grades/stages (37,38); mutation frequency low</td>
</tr>
<tr>
<td>DBC1 (9q32–33)</td>
<td>Deletion/methylation</td>
<td>LOH 60% all grades/stages (39,40)</td>
</tr>
<tr>
<td>TSC1 (9q34)</td>
<td>Deletion/mutation</td>
<td>LOH 60% all grades/stages (41–43); mutation ~13%</td>
</tr>
<tr>
<td>P TEN (10q23)</td>
<td>HD/mutation</td>
<td>10q LOH in 30–35% muscle invasive (44–46); mutation 17% high stage (47)</td>
</tr>
<tr>
<td>RB1 (13q14)</td>
<td>Deletion/mutation</td>
<td>10–15% overall (48–50); 37% muscle invasive</td>
</tr>
<tr>
<td>TP53 (17p13)</td>
<td>Deletion/mutation</td>
<td>70% muscle invasive (51–53)</td>
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Table II. Common regions of deletion detected by LOH analysis in transitional cell carcinoma

<table>
<thead>
<tr>
<th>Cytogenetic location</th>
<th>Frequency (%)</th>
<th>Association with clinical parameters</th>
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<tbody>
<tr>
<td>3p</td>
<td>48</td>
<td>Stage (54)</td>
</tr>
<tr>
<td>4p</td>
<td>22</td>
<td>None (55.56)</td>
</tr>
<tr>
<td>4q</td>
<td>24</td>
<td>High-grade/stage (56)</td>
</tr>
<tr>
<td>8p</td>
<td>23</td>
<td>High-grade/stage (57–61)</td>
</tr>
<tr>
<td>9q</td>
<td>60</td>
<td>None (62.63)</td>
</tr>
<tr>
<td>11p</td>
<td>40</td>
<td>Grade (64.65)</td>
</tr>
<tr>
<td>11q</td>
<td>15</td>
<td>None (65)</td>
</tr>
<tr>
<td>14q</td>
<td>10–40</td>
<td>Stage (66)</td>
</tr>
</tbody>
</table>

have profiled a series of tumours comprising all grades and stages. This has allowed genetic events to be related to tumour grade and stage and the majority of alterations studied to date have been found to be associated with high tumour grade and stage.

Molecular alterations in superficial UCC

Low-grade (G1–2) pTa tumours show few molecular alterations apart from deletions involving chromosome 9 and mutations of the FGF receptor 3 (FGFR3) (Table I). These tumours are often near-diploid with loss of chromosome 9 by far the most common cytogenetic finding (74). Similarly, loss-of-heterozygosity (LOH) analysis has revealed little difference from chromosome 9 LOH (Table II). LOH of 11p is found in ~40% of bladder tumours, including some pTa tumours, but is more common in tumours of higher grade and stage (65,75). The suggested target on 11p is CDKN1C (p57KIP2) (76). Comparative genomic hybridization (CGH) has identified other copy number changes including gain of 1q, 17 and 20q, amplifications of 11q and loss of 10q but none of these are frequent (Table III). Amplifications of 11q include the cyclin D1 gene (CCND1) which is involved in regulation of cell cycle progression from G1 to S phase via the Rb pathway (Figure 2).

As bladder cancer is a disease of the middle to late decades of life, it is predicted that multiple heritable changes are required for tumour development. Thus it is surprising that so few genetic changes have been identified in low-grade pTa tumours, which represent the major group at diagnosis. Epigenetic events represent an alternative type of heritable change and promoter hypermethylation of APC, p14ARF and RASSF1A has been described in the urine of almost all bladder cancer patients including those with low-grade/stage tumours (77). However, as for many other heritable changes, hypermethylation of several of these promoter regions shows significant association with tumour grade and/or stage (27,28). Indeed, one mechanism by which methylation can be increased is by upregulation of expression of DNA methyltransferase I, which occurs in cells with loss of Rb or p53 function (78,79), or metachronous tumours from the same patient generally show a striking identity in the genetic alterations found (72,80). LOH of chromosome 9 is the least divergent event, indicating that this is likely to be an early change, whilst other events occur during independent evolution of different tumour subclones (80). As relatively few common events have been identified in this group of tumours, efforts are in progress using expression and genomic microarray technology to identify other genetic or epigenetic events that may contribute to their development.
These genes share coding region in exons 2 and 3 but have distinct exons 1. The protein products are translated in different reading frames to generate two entirely different proteins, p16, which is negative regulator of the Rb pathway and p14ARF, a negative regulator of the p53 pathway (Figure 2). Both genes are commonly inactivated in UCC via homozygous co-deletion. Detection of homozygous deletion (HD) in tumour tissue by PCR-based techniques may be confounded by the presence of normal stromal and inflammatory cells and this may explain a lack of consensus on the relationship of 9p21 HD to clinical parameters in many published studies. To maximize the sensitivity of detection we recently used microdissected tumour tissue for HD assessment and did find an association with high-grade and stage (93). This will now require confirmation in a larger tumour series. LOH of 9p21 is as common in low-grade pTa as in invasive (≥pT2) UCC, and reduced copy number of 9p21 with or without LOH is present in ∼45% of tumours (93), indicating that as suggested by knockout mouse studies [reviewed in (94)] and in vitro experiments on mouse cells (95), haploinsufficiency of p16 and/or p14ARF may contribute to bladder tumour development.

Three genes on 9q are implicated; PTCH, the Gorlin syndrome gene, is within a small region of deletion at 9q22. Mutations of the gene are infrequent (37) but reduction in mRNA expression is common (38). At 9q33, a novel gene, DBC1, has been identified. HD has been found in a few tumours (92,96) and there is common transcriptional silencing by promoter hypermethylation (39,85). The function of DBC1 is not yet clear but ectopic expression can induce a non-apoptotic form of cell death (97) or delay in the G1 phase of the cell cycle (98).

The third gene, at 9q34, is TSC1. Germline mutation of TSC1 is associated with the familial hamartoma syndrome tuberous sclerosis complex (TSC). The TSC1 gene product hamartin acts in complex with the TSC2 gene tuberin in the PI3-kinase pathway to negatively regulate mTOR, a central molecule in the control of protein synthesis and cell growth (99) (Figure 3). Mutations of TSC1 are found in ∼13% of UCCs (41,42). Interestingly some of these are in tumours without 9q34 LOH, again indicating possible haploinsufficiency (41). Thus loss of one chromosome nine homologue, a common event in superficial bladder tumours, could possibly affect haploinsufficient genes on both 9p and 9q.

**FGFR3**

The most exciting recent finding in bladder cancer is mutation of the FGF receptor 3 gene (FGFR3) (13,100–102) (Table I). Mutation is strongly associated with low tumour grade and stage with up to 80% of low-grade pTa tumours showing mutation (100). Mutations have also been found in urothelial papilloma (103), a benign exophytic growth characterized by a covering urothelium that is indistinguishable from that of the normal urothelium and which may therefore represent a precursor to superficial UCC. The mutations found in UCC are confined to hot-spots in exons 7, 10 and 15 and all are predicted to cause constitutive activation of the kinase activity of the receptor (104). The same mutations are found in the germ-line in inherited dwarfism syndromes [reviewed in (105)] (Figure 4).

The consequences of FGFR3 activation in the urothelium are not yet confirmed but a likely consequence is activation of the MAPK and/or PI3-kinase pathways. Interestingly, Ras gene

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**Chromosome 9**

Chromosome 9 LOH is found in >50% of all bladder tumours regardless of grade and stage (75,81–82). Many UCCs have LOH of the entire chromosome, suggesting loss of function of tumour suppressor genes on both chromosome arms. Thus, identification of these genes is considered vital to aid understanding of disease pathogenesis and to provide useful clinical markers and targets. Critical regions of LOH have been mapped on both 9p and 9q. In small primary tumours, the frequency of small deletions appears higher, suggesting that initially small regions of LOH may develop and coalesce during tumour development (62,83–84).

Currently, one region of loss is mapped on 9p (9p21) and at least three regions on 9q (at 9q22, 9q32–q33 and 9q34) (38,62,85–87). Candidate genes within these regions are CDKN2A/ARF (p16/p14ARF) and CDKN2B (p15) at 9p21 (33,34,88–90), PTCH (Gorlin Syndrome gene) at 9q22 (37,38), DBC1 at 9q32–q33 (85,91–92) and TSC1 (Tuberous Sclerosis Syndrome gene 1) at 9q34 (41–43).

The CDKN2A/ARF focus on 9p21 encodes two proteins, p16 and p14ARF, both of which are key cell cycle regulators. The p14ARF locus encodes p16 and p14ARF that act as negative regulators of the Rb and p53 pathways, respectively. This interrelated signalling network is central to tumour suppression via the mechanisms of cell cycle arrest and apoptosis. Stimulation by mitogens induces cyclin D1 expression. Phosphorylation of Rb by CDK4-cyclin D1 complexes releases E2F family members to induce expression of genes required for progression into S phase. The cyclin D–CDK4 complexes also sequester p27 and p21 (data not shown). This allows formation of p53 and Rb pathways induced and this leads to cell cycle arrest via inhibition of cyclinE-CDK2, which reinforces the inactivation of Rb. p16 negatively regulates this process by interacting with CDK4. The p53 pathway responds to stress signals, e.g. DNA damage. p53 is altered as the result of p53-triggered cell cycle arrest via p14ARF. Grey shading indicates proteins involved reading frames to generate two entirely different proteins, p16, which is negative regulator of the Rb pathway and p14ARF, a negative regulator of the p53 pathway (Figure 2). Both genes are commonly inactivated in UCC via homozygous co-deletion. Detection of homozygous deletion (HD) in tumour tissue by PCR-based techniques may be confounded by the presence of normal stromal and inflammatory cells and this may explain a lack of consensus on the relationship of 9p21 HD to clinical parameters in many published studies. To maximize the sensitivity of detection we recently used microdissected tumour tissue for HD assessment and did find an association with high-grade and stage (93). This will now require confirmation in a larger tumour series. LOH of 9p21 is as common in low-grade pTa as in invasive (≥pT2) UCC, and reduced copy number of 9p21 with or without LOH is present in ∼45% of tumours (93), indicating that as suggested by knockout mouse studies [reviewed in (94)] and in vitro experiments on mouse cells (95), haploinsufficiency of p16 and/or p14ARF may contribute to bladder tumour development.

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mutations (HRAS, NRAS or KRAS2) and FGFR3 mutation have been found to be absolutely mutually exclusive events in UCC, suggesting possible biological equivalence (12). Mutation of either a Ras gene or FGFR3 was present in 82% of low-grade tumours, indicating that a common biological mechanism may be shared by virtually all superficial UCCs. Mutations of FGFR3 and TP53 are also somewhat mutually exclusive (106,107) but in this case, mutation of TP53 is associated predominantly with tumours of high-grade and stage, so it is hypothesized that these events are not equivalent, but rather are associated with and define the two distinct tumour groups.

Molecular alterations in invasive UCC

In addition to frequent chromosome 9 deletion, many genetic alterations have been reported in muscle invasive UCC (Tables I–III). These include alterations to known genes and genomic alterations for which the target genes are currently unknown. Karyotype studies have identified losses of 1p, 6q, 9p, 9q and 13q and gains of 5p in >15% of cases. CGH and LOH analyses have confirmed and extended these findings (Tables II and III).

Oncogenes

As for FGFR3 mutation in superficial UCC, several events identified in invasive tumours may activate the MAPK and/or the PI3-kinase pathways. ERBB2 (17q23), a receptor tyrosine kinase of the EGFR gene family, is amplified in 10–20% and overexpressed in 10–50% of invasive UCC (15,17,108,109). A similar situation exists in the case of the EGF receptor where 30–50% of invasive tumours overexpresses EGFR and this is associated with poor prognosis (110). In this case, only a very small percentage have gene amplification. Overexpression of epiregulin, a ligand for EGFR has recently been shown to be associated with tumour stage and with increased metastatic potential in a model system (111).

Ras genes are mutated in some invasive UCCs and there is no clear association with invasive rather than superficial disease. In vitro experiments on human tumour cells indicate that HRAS can upregulate EGFR expression and induce an invasive phenotype (112,113). However, in transgenic mice engineered to express mutant HRAS in the urothelium this leads to the development of superficial papillary tumours rather than muscle invasive tumours (114). Further studies in animal models may help to clarify whether HRAS can participate in the development of both major forms of UCC.

Some UCCs (4–6%) show amplification of MDM2 (12q14) (20). As MDM2 regulates p53 levels, its overexpression represents an alternative mechanism by which p53 function may be inactivated (Figure 2). Several immunohistochemical studies have shown upregulation of expression of MDM2 in UCC samples but there is no consensus on the relationship of this to tumour grade, stage or prognosis (115–117). MYC is upregulated in many bladder tumours (118). The mechanism for this is unclear. Although high-level amplification of 8q is found in some invasive UCCs, MYC does not appear to be the target for these. Nevertheless the common finding of additional copies of the whole of 8q may lead to overexpression. Alternatively, MYC may be transcriptionally activated by other molecular events in UCC e.g. stimulation of the MAPK pathway (Figure 3).

An amplicon identified on 6p in bladder tumours and cell lines (23–25) contains the E2F3 gene. Approximately 14% of muscle invasive UCCs have amplification of E2F3 (25). The E2F transcription factor family comprises seven genes, several of which are known to interact with and be regulated by Rb (Figure 2).

Tumour suppressor genes

Several tumour suppressor genes implicated in invasive UCC, including TP53, RB1, CDKN2A/ARF (also altered in many superficial tumours) and PTEN, are major players in other human cancers. The interconnecting pathways controlled by p53 and Rb that regulate cell cycle progression and responses to stress, processes that are almost universally deregulated in malignant cells [for review see (119)] (Figure 2), are commonly altered in invasive UCC. Mutation of TP53 is found in many muscle invasive tumours (51–53,120,121). Immunohistochemistry can detect p53 protein with increased half-life and as this identifies a significant proportion of mutant p53 proteins it has been commonly used as a surrogate marker for mutation (122). However it should be noted that many TP53 mutations (~20%) yield unstable or truncated proteins that cannot be detected in this way. There are also circumstances in which other alterations in the tumour cell result in upregulation of wild-type p53 protein. Comprehensive mutation
screening would provide the best data to relate to clinical parameters but to date a large series has not been examined in this way. The type and distribution of \textit{TP53} mutations found to date in bladder tumour samples can be found in the IARC \textit{TP53} Database (123). In several studies that have used p53 immunohistochemistry, p53 accumulation has been associated with adverse prognosis in all grades and stages of UCC (124–127). However, it still remains unclear whether it represents a useful prognostic marker and a recent meta-analysis of more than 3000 tumours has only indicated a small association between p53 positivity by immunohistochemistry and poor prognosis (128). Reduced expression of p21 which acts downstream of p53, is associated with disease progression (129,130). The \textit{RB1} gene has not been screened for small mutations in UCC but some HDs, LOH of 13q14 and loss of Rb protein expression have been detected in tumours of high-grade and stage (48–50,131).

As indicated above, the locus encoding p16 and p14\textit{ARF} is commonly deleted in UCC of all grades and stages. These proteins interact with and link the Rb and p53 pathways (Figure 2) and due to the multiple regulatory feedback mechanisms that operate in these pathways (132), inactivation of both of these together is likely to provide further freedom from the G\textsubscript{1} checkpoint than conferred by either p53 or Rb inactivation alone. It is predicted that tumours with p53 and Rb or p16 loss will be more aggressive than those with either p53 or Rb loss alone. Rb and p53 have been assessed together in several clinical studies and this prediction is borne out. Altered p21 expression both alone and in combination with other events also represents a significant risk factor (129,130). To date an assessment of all genes known to be involved in the G\textsubscript{1} checkpoint has not been carried out on a single patient series but such an analysis may achieve even greater predictive power.

The cyclin D1 gene \textit{CCND1} is amplified in some bladder tumours, both superficial and invasive. However, the frequency of amplification is insufficient to explain all cases with overexpression. It is likely that overexpression in many cases is the indirect result of other alterations e.g. activation of the MAPK or PI3-kinase pathways (Figure 3). To date there is no clear consensus on the clinical significance of overexpression. Interestingly, cyclin D1 is a possible target of the Wnt/\beta-catenin pathway and recently some high-grade bladder tumours with mutations in \beta-catenin were reported (133). This represents an alternative mechanism for cyclin D1 activation in these tumours and is accompanied by myc overexpression and a more aggressive phenotype than the overexpression associated with gene amplification, found in low-grade tumours with papillary architecture (134).

\textit{PTEN} \textit{PTEN} (phosphatase and \textit{ten}sin homologue deleted on chromosome ten) maps to 10q23, a region of common LOH in UCC of high-grade and stage (44,46,135). \textit{PTEN} has both lipid and protein phosphatase activity, a key substrate being the signalling lipid PtIns(3,4,5)P\textsubscript{3} a major product of PI3-kinase which is activated by various tyrosine kinase receptors. Thus

Fig. 4. FGFR3 mutations found in bladder cancer. Positions of mutations in bladder cancer are shown in relation to gene structure. Codons are numbered according to the FGFR3 IIIb isoform. When present in the germline, all of these mutations apart from one (K652T) are associated with skeletal dysplasia syndromes. Thanatophoric dysplasia types I and II are lethal forms of skeletal dysplasia. Achondroplasia is the common (non-lethal) form of dwarfism. SADDAN, severe achondroplasia with developmental defects and acanthosis nigricans. Data for bladder cancer is from refs (13,14,100,102,103,106,167–173) and shows the distribution of 784 reported mutations.
PTEN is a negative regulator of this signalling pathway, which affects cell phenotype in various ways including effects on proliferation, apoptosis and cell migration (136) (Figure 3). Heterozygous knockout mice (Pten+/−) show widespread proliferative changes, suggesting that loss of one allele in tumours may provide an advantage at the cellular level. Mutation screening in UCC has revealed some mutations of the second allele in tumours with LOH and HD in some bladder cell lines (45,47,137). Re-expression experiments in bladder tumour cells that lack PTEN have revealed an effect on tumour cell chemotaxis and anchorage independent growth (138) and it has also been shown that PTEN re-expression suppresses tumour growth in vivo (139,140).

Loss of PTEN leads to PI3-kinase pathway activation with high levels of phosphorylated AKT. As discussed above, the TSC1 product hamartin also acts in the PI3-kinase pathway (Figure 3), possibly providing an alternative mechanism of pathway activation. Although bladder tumour cell lines with known PTEN mutation and some bladder tumour tissues have increased phosphorylation of AKT (141), a large survey of cell lines and tumour tissues has not yet been carried out. Possibly, constitutive activation of the pathway occurs in many UCCs and in the future it will be important to examine other possible mechanisms of activation.

Other genetic changes in invasive UCC

Large numbers of genomic changes have been detected by karyotyping, CGH and LOH analyses in muscle invasive UCC. Numerically common are losses of 2q, 5q, 6p, 9p, 9q, 10q, 11p, 18q and Y (Tables II and III). There is also much detailed information from recent array-based CGH and SNP array analyses which identify some novel regions of copy number change and/or allelic imbalance in invasive UCC (73,142,143). Gains of 1q, 5p, 8q and 17q are frequent and several high level amplifications have been found. To date the target genes within most of the regions of gain or high level amplification have not been conclusively identified.

This group of invasive tumours displays genetic instability with rapid and major genetic divergence in related tumours from the same patient and this is commonly chromosomal instability (CIN) rather than microsatellite instability (MIN) (144). The genetic differences between minimally invasive (pT1) and more deeply invasive tumours (≥pT2) are not significant, suggesting that tumours with the ability to break through the basement membrane are aggressive lesions. Although T1 tumours often have good clinical outcome, this may reflect complete resection rather than lack of tumour aggression. Certain genetic changes including gains of 3p22–25 and 5p and losses of 4p11–15, 5q15–23, 6q22–23 and 10q24–26, appear to be associated with tumour progression in this group (68). In one CGH study, muscle invasive tumour samples and paired metastatic samples were compared but no significant metastasis-associated markers were identified (70).

CIS

CIS is a difficult lesion to obtain as a fresh tissue sample due to its fragility. By definition, CIS has normal urothelial thickness and commonly the cells, which are highly anaplastic, are only weakly adherent and tend to dissociate during cystoscopy (Figure 1c). Thus most specimens are only recognized retrospectively in paraffin-embedded samples and only a few studies have attempted to assess genetic changes in such lesions. An initial study by Spruck et al. (145) suggested that dysplasias and CIS had a high frequency of TP53 mutation but a relatively low frequency of chromosome 9 loss. A subsequent study (146) found chromosome 9 loss and a range of alterations similar to those reported in muscle invasive UCC. These apparently discrepant results may be explained by a more recent study (147) which indicates significant differences in CIS that is found in isolation (primary CIS) and CIS that occurs in association with a papillary tumour. Chromosome 9 loss was infrequent in primary CIS and common in CIS associated with synchronous carcinoma. If confirmed, this may indicate two distinct forms of CIS with different developmental pathways. CIS found in association with high-grade superficial UCC and containing chromosome 9 deletion could represent a precursor of the associated papillary tumour.

Signalling pathways in UCC

Do key signalling events define the superficial and invasive tumour groups? The finding of FGFR3 or Ras gene mutations in the vast majority of superficial tumours suggests that these tumours share changes in pathway activation. Similarly the common inactivation of Rb and p53 pathways only in invasive tumours and the much lower frequency of FGFR3 mutations in this group may indicate a distinct signalling status. As illustrated in Figures 2 and 3, several known alterations could potentially activate both the MAPK and PI3-kinase pathways and inactivate the Rb and p53 pathways. Whilst there is some evidence for activation of the PI3-kinase pathway in tumour samples (141) via the known mutation of PTEN and TSC1, there is as yet no direct information on the status of the MAPK pathway. Interestingly, in bladder tumour cell lines, which in terms of their origins and genetics are representative of invasive tumours, this pathway is not highly constitutively activated. This contrasts with the situation in proliferating normal urothelial cells in culture which have been reported to show high levels of ERK phosphorylation (148). Direct studies of MAPK pathway status in tumours are currently lacking but if active, the known cross-talk between the MAPK and PI3-kinase pathways via Ras and ERK (Figure 3) firmly places the PI3-kinase pathway at centre stage and indicates the likely clinical utility of inhibitors of this pathway in UCC treatment.

It may be postulated that excessive stimulation of these pathways in the absence of additional molecular changes is likely to lead to induction of a stress response via various feedback loops. For example high levels of mutant Ras signalling in normal cells induces cell cycle arrest via activation of p53. Similarly, high levels of E2Fs and MYC may induce p53 dependent cell cycle arrest or apoptosis via p14ARF.

The effect of high level FGFR3 signalling is not yet known but possibly this is less detrimental to the normal urothelial cell than other oncogenic stimuli and may be well-tolerated as an early event. The unique association of FGFR3 mutation with low-grade superficial UCC is of great interest and possibly provides the best molecular prognostic indicator identified to date. It has been suggested that FGFR3 mutation may have a protective effect and prevent tumour progression. It is essential that its role in determining tumour behaviour is now examined in detail.
Tumour clonality and timing of events

Multifocality and frequent recurrence is characteristic of urothelial tumours. The macroscopically ‘normal’ urothelium in many cases shows areas of microscopic dysplasia (149) so that it is easy to envisage how new lesions develop after resection of the primary tumour. There has been much discussion of the clonality of bladder tumours. One possibility is that the entire urothelium is unstable and many different clones of altered cells are present that give rise to polyclonal tumours, the so-called ‘field effect’. Most studies have found only monoclonal tumours. The presence of shared genetic changes in all tumours resected from individual patients suggests that these are related lesions that have evolved from a single altered cell clone. Divergence between the genetic changes found in such related tumours has been used to determine likely timing of events and loss of chromosome 9 is predicted to be an early event on this basis (80). However, there are some examples of more than one unrelated monoclonal tumour in the same bladder (oligoclonality), and this is not surprising, given the association of TCC risk with smoking and the pan-urothelial carcinogenic insult associated with this [reviewed in (150)].

Recently, attempts have been made to apply bioinformatic modelling to define possible genetic pathways of UCC development (151,152). Some associations of events have been identified. For example, Hoglund *et al.* using principle component analysis of data obtained via conventional cytogenetic analysis of 200 UCCs identified two potential cytogenetic events, one initiated by $-9$, followed by $11p-$ and $1q+$ and a second initiated by $+7$ followed by $8p-$ and $4q+$. The latter group appeared to contain more aggressive tumours (T1–T3) whereas the former contained Ta-T2 tumours (151). More recently, Bulashevska *et al.* (152) used a Bayesian network model to analyse LOH data for 17 chromosomes in 123 papillary UCCs (all grades). Such an analysis will not detect increased copy number and hence it is difficult to directly compare the findings with those of Hoglund *et al.* (151). However, the network obtained showed $9p-$ and $9q-$ as the most probable primary event with $8p-$ and $17-$ as major subsequent events that lead to progression. Other events showed relationship to one or either of these e.g losses of 1q, 18q and 10q were related to $8p$ loss and $5q$ loss to $17p$ loss. A more recent analysis (153) based on cytogenetic data confirms several of these associations and predicts two pathways that show distinct lesions but ultimately converge, one initiated by $+7$ and the other by $-9$.

Based on molecular and histopathological observations, a model for the molecular pathogenesis of UCC has developed (Figure 5). Almost certainly this is too simple but it does provide a useful anchor for molecular studies. Mutation of *FGFR3* defines the large group of superficial tumours. *FGFR3* and *TP53* mutation are each confined to one of the two major subgroups of UCC (106,107) and currently are the best molecular markers for these groups.

There are several gaps in our current understanding. First there may be more alterations in low-grade papillary superficial UCC that remain to be discovered. Another outstanding question is what the significance of pT1 tumours is. Are these merely muscle invasive tumours caught in their journey towards the muscle or do they represent a distinct group? Genetic findings tend to support the former conclusion but this is not proven. No significant differences have yet been found between muscle invasive UCC and the metastases that develop from them. Possibly this reflects the early migration of cells to distant sites without the requirement for additional changes or possibly there are determinants of progression and metastasis yet to be identified. Finally, the critical question concerning possible differences in primary and UCC-associated dysplasia/CIS (147) which may confirm the existence of a third pathway to urothelial neoplasia requires careful examination of more samples.

Information from global expression and genomic profiling

Global profiling via expression and genomic microarray technologies may provide answers to some of these outstanding questions. Detailed discussion of findings is beyond the scope of this commentary but some findings are worthy of comment.

Expression arrays have been used to classify bladder tumours and provide gene expression patterns that define the major histopathological subtypes (154–158). Encouragingly a classification set of transcripts recently identified by Blaveri *et al.* (157), when tested on data from a previous study (159) showed successful classification into superficial and muscle invasive tumour groups.

Several reports have attempted to go beyond simple classification to identify subsets of tumours within grade/stage classes and to predict outcome. For example, a study of Ta
tumours generated a ‘molecular classifier’ that provided predictive information on risk of recurrence (159). Two studies have reported comparisons of groups of superficial tumours that did or did not progress (160–161). In the first (161), a classifier was derived from a test series of tumours and was then tested on an independent set of 74 tumours. Although the positive predictive value was low (0.3) the negative predictive value was high (0.95) (only 1 of 20 tumours with the non-progression signature showed later progression), indicating that this may have clinical application in excluding such patients from intensive cystoscopic follow-up. In the second study (160), expression profiles discriminated between Ta, Tis, T1 and T2 tumours and showed that in general T1 tumours were more similar to T2 than to Ta tumours. A predictor of progression was built that correctly classified 33/42 samples (sensitivity 85.7%; specificity 71.4%).

A study of superficial tumours with or without associated CIS, a known high risk predictor, showed that these could be accurately classified by expression profile and a 16 gene CIS classifier was derived that performed well. Interestingly ‘normal’ mucosa from cystectomy specimens with adjacent CIS was found to contain the CIS signature, indicating that in bladders with CIS, there is widespread urothelial alteration (162). Whether such gene expression alterations are reversible or whether they indicate widespread heritable alteration in the urothelium is not known. A critical experiment will be to determine the genetic profiles of superficial papillary tumours and associated CIS to assess their relationship. A striking feature of this study was that the tumours with adjacent CIS had distinct expression profiles from the muscle invasive tumours analysed in contrast with the findings of a previous study in which such a relationship had been found in a small number of tumours (159). Although the numbers analysed are small, this allows the possibility that two populations of muscle invasive tumours may exist, one with primary CIS as a direct precursor and another without, possibly arising directly from a high-grade superficial papillary tumour, the third pathway discussed above (Figure 5). Muscle invasive tumours with known outcome have not yet been studied in large numbers but Blaveri et al. (157) were able to classify invasive tumours with associated good (survival \( \geq 18 \) months) or bad (survival \( < 18 \) months) prognosis with 78% success using a 24 gene signature.

Although firm conclusions cannot yet be drawn about critical markers for classification and prediction of progression, it is re-assuring that common genes have emerged from several expression studies as potential markers for particular subgroups of tumours. These include cathepsin E (CTSE) which is more highly expressed in normal urothelium and superficial tumours (157,160) and FABP4, high expression of which was present in normal urothelium and associated with superficial tumours that progressed but was reduced in muscle invasive tumours (160) and in CIS (162).

Few studies to date have carried out global genomic profiling of bladder cancer. Two possible approaches are possible. Array-based CGH examines DNA copy number across the genome, typically by hybridization of differentially labelled tumour and normal DNAs to spotted BAC arrays covering the genome at high density. SNP array analysis, compares single nucleotide polymorphisms throughout the genome in matched normal:tumour DNA samples by hybridization to oligonucleotide arrays. Both LOH and copy number alterations can be scored using this latter platform, though currently copy number analysis does not appear as accurate as that obtained using spotted BAC arrays. To date SNP array analysis has been used more extensively on bladder cancer samples (142,163–165). Consistency with microsatellite-based LOH analysis has been confirmed and the method has been used successfully to detect tumour cells in urine. The high resolution of the technique has allowed some novel chromosomal regions of LOH to be identified (142). Similarly, array-based CGH has provided high resolution copy number profiles and in particular has allowed regions of high level amplification to be defined more precisely than is possible by classical CGH (24,143).

Conclusions

The past few years have seen an exponential accumulation of molecular information on UCC. Encouragingly, expression array analyses, which have provided orders of magnitude more information than has ever been acquired on gene expression in this disease, have largely confirmed that groupings based on tumour grade and stage are biologically valid. These studies and some of the more simple genetic analyses have also indicated that some subgroups such as papillary tumours that progress or have associated CIS do indeed form distinct groups at the molecular level. Doubt remains about the precise genetic relationships of putative precursor lesions, low- and high-grade papillary and muscle invasive UCCs. However, it comes as no surprise that there are now several lines of evidence for more complexity in pathogenesis than can be explained by a simple two-pathway model. Possibly both ‘Jekyll and Hyde’ and ‘chalk and cheese’ are represented here. The challenge will be to recognize these scenarios and to predict clinical phenotype in every patient. Undoubtedly, the application of array-based genomic approaches used in combination with expression profiling and high throughput mutation scanning will yield a wealth of information in the future that may answer some of the outstanding questions about the genetic relationships between different histopathological entities. Key to this will be appropriate selection of tissues for study and there will be great advantages if archival samples from clinical trials can be used. As critical molecular events are identified it will also be essential to test their effects on human urothelial cell phenotype. Here the development of relevant tissue culture models combined with appropriate xenografting studies as recently used to elucidate the requirements for transformation of other epithelial cell types (166), may provide critical confirmatory information.

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References

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Molecular subtypes of bladder cancer


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