Tumor suppressor loci in bladder cancer

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1. ABSTRACT

Large numbers of genetic and epigenetic alterations have been identified in bladder cancer in recent years. Many of these affect the function of tumour suppressor genes (TSGs), leading to partial or complete loss of protein expression or function with varied phenotypic consequences. Some of the genes implicated such as *TP53* and *RB1* are major players in many other tumour types. Others, particularly some on chromosome 9, show bladder-specific involvement. Other TSGs of relevance to bladder tumour development are predicted by the finding of common physical deletion or LOH in specific regions of the genome. This review summarises the approaches that have been used to identify bladder tumour suppressor genes, the current state of knowledge of the genes involved in this disease, their relationship with specific clinical features and some possible therapeutic applications.

2. INTRODUCTION

2.1. Bladder cancer

Bladder cancer is a common disease affecting more than 12,500 patients in the UK and an estimated 63,000 in the USA each year with 5,000 and 13,000 deaths respectively (1, 2). Here the most common form of the disease (>90%) is UCC. Worldwide it is estimated that >300,000 new cases occur each year, with the highest incidence in industrialised countries and areas where infection with the parasite *Schistosoma haematobium* is endemic. In the middle East where infection with this parasite is common, squamous cell carcinoma of the bladder predominates and in some areas e.g. Egypt, bladder cancer is more common than all other types of cancer. Elsewhere, 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 and USA 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 percent 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.

The various genetic and epigenetic changes that represent the primary heritable changes present in a cancer and the resulting wide range of secondary changes in gene expression represent molecular markers that can be used to advantage in a clinical setting in a number of ways. These include application for classification of tumours at diagnosis, to monitor the course of disease and by known associations to predict prognosis and/or response to therapy. These changes also provide the key to development of individualised therapies based on the molecular profile of the tumour. Examples include the development of drugs that inhibit specific targets, reagents that can potentiate the immune response to specific antigens or gene therapy strategies that replace the function of damaged genes or utilise specific molecular features of a tumour to elicit cytotoxic effect. Our understanding of the molecular changes underlying the development of bladder cancer has progressed rapidly during the past 15 years. Not only do we now have a long list of genetic and gene expression changes found in tumours but also a proposed model for the pathogenesis of UCC based on molecular information.

Studies of the molecular mechanisms of cancer development have revealed that not only are multiple heritable events required to generate solid tumours in adults but that there are repertoires of genes that function within the same pathway, alterations of which represent mutually exclusive alternatives to fulfil the same function in the tumour cell. In some cases oncogenes or tumour suppressor genes that act in the same pathway may represent valid alternatives. This review will focus on what is known about TSGs involved in the development of bladder cancer. However, some brief description of the key oncogenes involved is included to provide a more complete view of the molecular pathogenesis of the disease and to identify situations where either a TSG or oncogene may fulfill the same function.

2.2. Tumour suppressor genes: definition
Tumour suppressor genes encode proteins whose loss of expression or function can contribute to the process of tumorigenesis. Two major classes of tumour suppressor gene have been defined (4). The so-called "gatekeeper" genes are those whose loss is rate-limiting and whose re-constitution in a tumour cell leads to reversion of phenotype. Such genes appear to carry out pivotal control functions, which may be tissue specific. Examples of this type include *APC*, which is lost early in the development of colorectal cancer and is mutated in the germline in familial adenomatosis coli, *RB1,* which is mutated in familial retinoblastoma and involved in a wide variety of sporadic

malignancies and *PTEN* which is also involved in both familial and sporadic cancers. Each of these genes controls vital signalling events within the cell. This type of tumour suppressor gene commonly acts in a recessive manner at the cellular level as demonstrated by early cell fusion experiments between normal and tumour cells which indicated a dominant function in the normal cells that was able to suppress markers of transformation in the tumour cells with which they were fused.

The second class of TSG is the "caretaker" or "stability" gene. Such genes are involved in maintaining genomic integrity and these include DNA repair genes and genes involved in chromosomal integrity and segregation. When the function of these genes is lost, this leads to increased mutation and genomic instability. Re-constitution of such genes in a cancer cell can have no effect on phenotype. In some cases tumour suppressor genes can play a role in both types of process and these are defined as both caretaker and gatekeeper e.g. *TP53*.

2.2.1. Mechanisms leading to loss of function of tumour suppressor genes
Loss of function of tumour suppressor genes can be achieved by a number of mechanisms during tumour development. For many TSGs including many gatekeepers that behave in a recessive manner, complete loss of function is required for phenotypic effect. The first TSG identified, *RB1* was found following studies of children with inherited susceptibility to retinoblastoma. In these cases, a germline deletion of the 13q14 region was followed by a second somatic event detected in the tumours that developed. This led Knudson to propose a "two hit" hypothesis for TSG inactivation in which both maternal and paternal alleles of the gene must be silenced (5). In sporadic cancers such as bladder, both events need to occur in a somatic cell. Mechanisms of loss may be genetic events such as deletions and small mutational events or epigenetic silencing via methylation of the promoter. Such events can occur in any combination to affect the two alleles of the gene. Examples are illustrated in Figure 1.

2.2.2. Haploinsufficiency
In recent years it has become apparent that reduction of expression of some genes, as caused by loss of function of a single allele, may have important consequences that can contribute to cancer development. This is termed haploinsufficiency. If loss of expression from one allele confers a selective advantage on a cell that allows clonal expansion within the normal tissue, this may increase the population of altered cells that are available for a second hit. Some elegant experiments using transgenic mice have recently provided evidence for haploinsufficiency of several TSGs implicated in human cancer e.g. *Pten, Tsc1* and *Cdkn1b* (reviewed in (6)).

2.3. Methods used to detect tumour suppressor loci in bladder cancer
As loss of one allele by deletion is a common mechanism for TSG inactivation, simple observation of chromosomal losses and deletions in tumour samples has provided good evidence for the location of relevant TSGs.
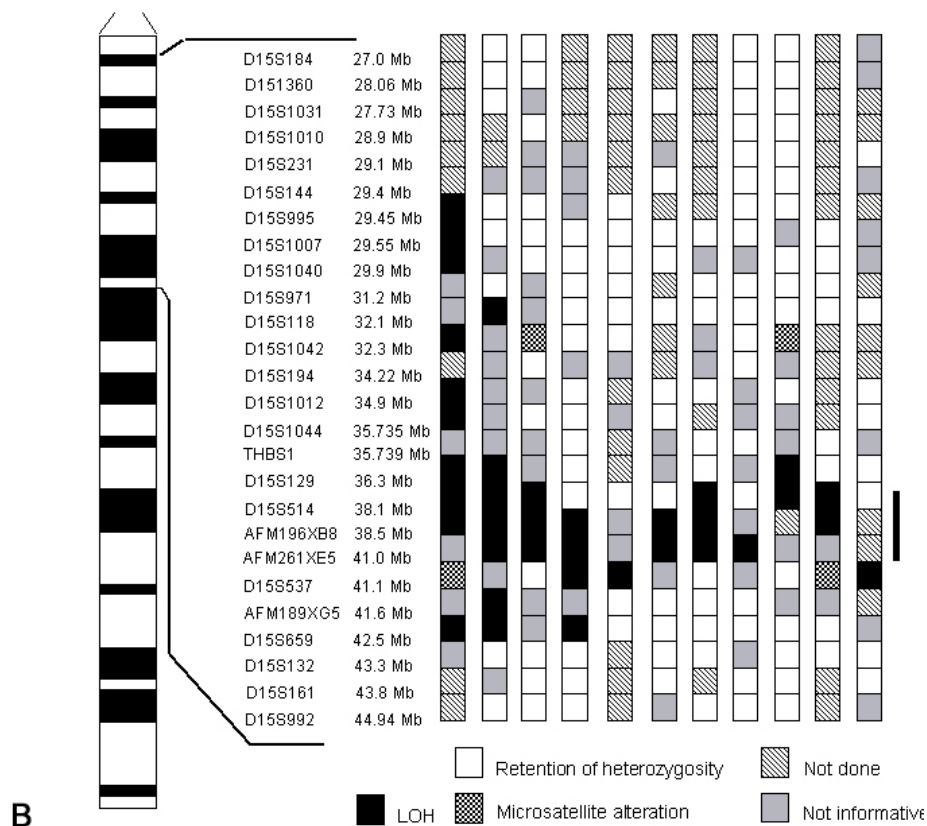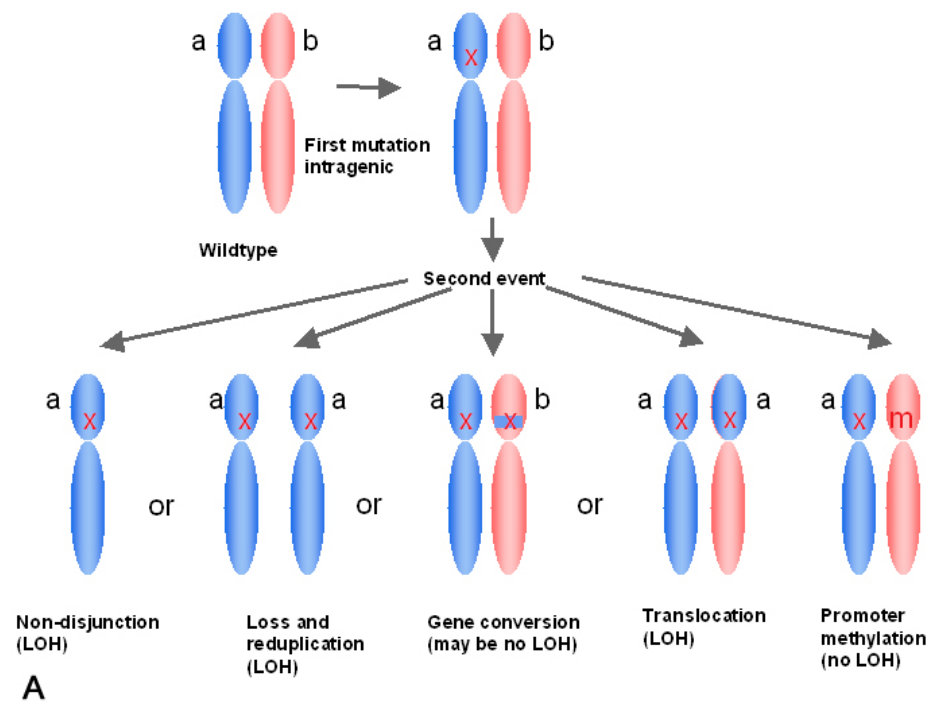
Figure 1. A. Possible mechanisms for biallelic inactivation of tumour suppressor genes in a somatic cell. Mutations that generate proteins that act in a dominant–negative manner and mutations in haploinsufficient genes would not require inactivation of the second allele. B. Example of tumour deletion maps for a region on 15q obtained by microsatellite-based LOH analysis. Each vertical bar represents a single tumour. Location of a critical region of deletion in which a candidate tumour suppressor gene may exist is indicated by solid line at right.

Figure 2. Staging of urothelial cell carcinoma.

In UCC, common cytogenetic events include -9 or 9q-, +7, 1p-, 1q-, 5q-, i(5p), 11p-, 6p-, 6q-, 17p-, 2q-, 3p-, +8, +11, 21q- and –Y (For review see (7)). The finding of monosomy 9 in bladder tumours of all grades and stages implicates 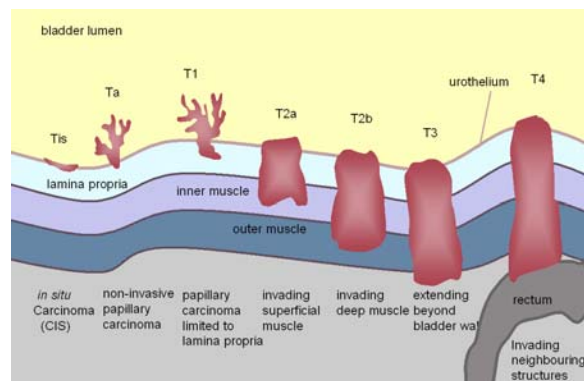chromosome 9 as the likely location of bladder TSGs that may be involved early in the transformation process and indeed this has been borne out by recent molecular analyses (see below).

The availability of information about polymorphisms in the human genome allows the tumour genome to be compared with constitutional DNA from the same individual. Thus for polymorphic loci for which a patient is heterozygous (informative), loss of one allele in the tumour may indicate a deletion event at that location in the genome. Such LOH studies provide valuable information on the predicted location of many TSGs in sporadic cancers. The recent identification of many highly polymorphic markers that can be assessed by PCR techniques, has led to precise mapping of many TSG loci in sporadic cancers including bladder (Figure 1). Where frequent LOH is identified, it is predicted that a target gatekeeper gene within the region will have sustained a second inactivating event. Sometimes this may be a second deletion leading to compete loss of all or part of the gene, known as homozygous deletion (HD). It is not common to find large homozygous deletions, even in highly aggressive tumour cells, presumably because loss of many genes is detrimental. Thus these events tend to be small. Homozygous deletions of the *CDKN2A* locus on 9p21 are common in several types of cancer including bladder (see below) and these may be identified by simple PCR-based techniques provided that adequate care is taken to ensure that tumour DNA samples contain virtually no contaminating normal DNA which would lead to a false negative result. More commonly, the second hit is a mutation which may be a point mutation affecting a residue that is essential for gene function or may generate a termination codon or a small deletion or insertion that leads to a frameshift and premature termination of the protein. Having identified candidate genes via LOH analysis, mutation screening of the coding sequence of the candidate genes is commonly undertaken to search for small inactivating mutations. The finding of a mutation that is predicted to alter protein function as a clonal event in a tumour indicates a possible tumour suppressor function.

The finding of many tumours with mutations in the same gene provides compelling evidence. Functional studies and knockout studies in mice usually follow to confirm a tumour suppressor role.

Comparative genomic hybridisation (CGH) analysis has been used to identify genomic regions of DNA copy number change. This technique uses differential fluorescent labelling of tumour and normal DNA and hybridisation to either a normal metaphase spread (conventional CGH) or to genomic clones or oligonucleotides immobilised on glass slides (array-based CGH) to estimate relative copy number in specific genomic regions. Much information has come from conventional CGH (8-12). In particular, several regions of high level DNA amplification have identified the location of potential oncogenes. With the advent of array-based approaches, resolution is now improved greatly and many novel high-level amplifications and small deletions including homozygous deletions have been finely mapped (13-15).

A common mechanism by which TSG expression is lost is promoter methylation (epigenetic silencing). The targets for methylation are cytosine residues in the dinucleotide CpG (reviewed in (16)). Methylation can be detected using a variety of methods but the most common involve bisulfite treatment of test DNA which converts non-methylated cytosines to uracil. This is then detected by sequencing, where non-converted C residues in a CpG dinucleotide are deemed to have been methylated, or by PCR using primers specific for converted or non-converted DNA.

Finally, as TSGs involved in other cancers have been identified, many known genes have been directly screened for mutation as candidates for involvement in bladder cancers.

3. TUMOUR CLASSIFICATION

Bladder tumours are classified using the TNM (Tumour, Nodes, Metastasis) classification system to describe tumour spread (stage) (17) and the WHO histological classification (18) to describe tumour differentiation (urothelial, squamous, adenocarcinoma etc) and degree of cellular atypia or dysplasia (grade). A diagrammatic representation of tumour staging is shown in Figure 2. More than 70% of bladder tumours at presentation are non-invasive tumours that have not penetrated the basement membrane (Ta).

In the past, bladder cancer has commonly been categorised into "superficial" (Ta, T1, CIS) and "invasive" (≥T2). In light of current genetic data, this subdivision appears inappropriate and current thinking is that low grade superficial papillary tumours (pTa G1/2) should be considered as distinct from those tumours that have penetrated the basement membrane and invade the submucosa (pT1) and from the high grade lesion carcinoma *in situ* (CIS), all of which have been grouped together in the past. These latter are high-risk lesions that commonly progress to invade muscle (19). High grade Ta tumours

Table 1. Common regions of deletion detected by LOH analysis in urothelial cell carcinoma

Cytogenetic location	Frequency	Association with clinical parameters	Reference
3p	48%	Stage	121
4p	22%	None	135, 136
4q	24%	High grade/stage	136
8p	23%	High grade/stage	110, 112, 114
9q	60%	None	27, 137
11p	40%	Grade	22, 138
11q	15%	None	22
14q	10-40%	Stage	139
15q	39%	None	140

Table 2. Regions of underrepresentation identified by CGH in urothelial cell carcinoma [1]

Tumour stage	Losses
Ta	9p, 9q, Y
T1	2q, 4p, 4q, 5q, 6q, 8p, 9p, 9q, 10q, 11p, 11q, 13q, 17p, 18q, Y
T2-4	As for T1 + 15q

[1] Data from:(8, 9, 23, 141-143).

(pTaG3) are also at increased risk of progression to invasion and this is reflected in a spectrum of molecular changes and genetic instability similar to that seen in T2 tumours. A grouping into genetically stable (low grade pTa) and genetically unstable (T1, CIS, high grade Ta), high-risk superficial tumours is therefore considered more appropriate.

4. DISTINCT MOLECULAR ALTERATIONS IN DIFFERENT TUMOUR SUB-GROUPS

4.1. Low grade superficial tumours

Few molecular alterations have been identified in low grade (G1-2) pTa tumours. Only 2 alterations are found at high frequency; deletions involving chromosome 9 and mutations of the FGF receptor 3 (*FGFR3)*. Karyotypic analysis reveals that these tumours are often near diploid and loss of chromosome 9 is the most common cytogenetic finding (20). Apart from this, only loss of the Y chromosome has been found at significant frequency. LOH and CGH analysis has revealed little apart from chromosome 9 LOH (Tables 1 and 2). 11p LOH is found in ~40% of bladder tumours, including some pTa tumours, though there appears to be a higher frequency in tumours of higher grade and stage (21, 22)(Table 1). CGH analysis has identified a few other copy number changes such as gain of 1q and 17 and some amplifications of 11q, but none are frequent. 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.

Low grade pTa tumours are relatively genetically stable and studies of synchronous or metachronous tumours from the same patient show a striking identity in the genetic alterations found (23, 24). Chromosome 9 LOH is the least divergent event and other genetic events differ in different tumours from the same patient. This indicates that LOH of chromosome 9 is likely to be an early change whilst other events occur during independent evolution of different tumour sub-clones (24).

4.2. High grade superficial and invasive tumours

Muscle invasive UCC is genetically complex and unstable. At the cytogenetic level these tumours show genomic instability with aneuploidy and many chromosomal re-arrangements. When LOH is assessed they show LOH at multiple loci (Table 1). Similarly, CGH commonly shows multiple copy number alterations in the same tumour (Table 2). Many known genes have been assessed for involvement in bladder cancer and for virtually all of these, mutation or other alterations are more common in muscle invasive tumours than in low grade superficial tumours (Table 3). Some of the genes known to be involved are common players in many human epithelial malignancies e.g. *TP53, RB1, PTEN*. In addition there are many genomic regions identified via CGH and other techniques that show common alteration but within which candidate genes have not yet been identified. Muscle invasive bladder cancer shows a high degree of genomic instability. In contrast to the low grade superficial tumours, there may be considerable diversity at the genetic level in lesions taken from different regions of the bladder or at different times during the course of the disease.

5. CHROMOSOME 9 LOCI

Chromosome 9 LOH is found in more than 50% of bladder tumours of all grades and stages (21, 25, 26). The finding that many UCCs have loss of the entire chromosome, commonly with reduplication of the retained homologue, indicates that loss of function of tumour suppressor genes on both chromosome arms may contribute to tumour development. Much effort has been made to identify these target genes, as this is considered pivotal to understanding the pathogenesis of the disease and providing useful clinical markers and targets. Tumours with regions of LOH affecting only part of the chromosome have been used to map critical regions of LOH on both 9p and 9q. Where small primary tumours have been studied, the frequency of smaller deletions appears higher, suggesting that initially small regions of LOH may develop and coalesce during tumour development (27-29). Loss of a copy of chromosome 9 has also been visualised by fluorescence *in situ* hybridisation (FISH) in urothelial dysplasia and in morphologically normal urothelium in patients with bladder cancer (30). This provides additional evidence that chromosome 9 loss is an early event in UCC development.

Table 3. Alterations of known tumour suppressor genes in urothelial cell carcinoma

	Tumour suppressor genes	
INK4A-ARF (9p21)	Homozygous deletion/ methylation/mutation	20-30% high grade/stage (37, 38, 46) LOH 60% all grades/stages immortalisation *in vitro* (144)
RB1 (13q14)	Deletion/mutation	10-15% overall (77, 89, 90) 37% muscle invasive
TP53 (17p13)	Deletion/mutation	70% muscle invasive (70, 72, 80) high grade and stage
PTEN (10q23)	Homozygous deletion/mutation	10q LOH in 35% muscle invasive (97, 99) 6.6% superficial
PTC (9q22)	Deletion/mutation	LOH 60% all grades/stages (31, 40) Mutation frequency low
TSC1 (9q34)	Deletion./mutation	LOH 60% all grades/stages (43-45) Mutation frequency low
DBC1 (9q32-33)	Deletion/methylation	LOH 60% all grades/stages (32, 145)
*DCC/SMAD (18q)	Deletion	LOH 30% high grade/stage (146) No mutation analysis to date

Currently, one region of loss is mapped on 9p (9p21) and at least three regions on 9q (at 9q22, 9q32-q33 and 9q34) (27, 31-34). Candidate genes within these regions are *CDKN2A/ARF* (p16/p14^{ARF}) and *CDKN2B* (p15) on 9p21 (35-39), *PTCH* (Gorlin Syndrome gene) on 9q22 (31, 40), *DBC1*(a novel gene) on 9q32-q33 (32, 41, 42) and *TSC1* (Tuberous Sclerosis Syndrome gene 1) on 9q34 (43-45) (Figure 3).

5.1. *CDKN2A/ARF*

The *CDKN2A/ARF* locus on 9p21 encodes 2 proteins, p16 and p14^{ARF} both of which are key cell cycle regulators (Figure 4). These genes share exons 2 and 3 but have distinct exons 1. The protein products are translated in different reading frames to generate two entirely different proteins one of which, p16, plays a key role in the Rb pathway and the other, p14^{ARF}, acts in the p53 pathway (Figure 5). Many studies have now shown that this locus is commonly inactivated in UCC via HD (36-38, 46). In the same region of 9p21 is the related gene *CDKN2B* encoding the cell cycle regulator p15 and in many cases, though not all, this gene is also homozygously deleted. Point mutations of p16 are infrequent (38, 47, 48) but hypermethylation of the promoter is found as a mechanism of inactivation of the second allele, though there is controversy over the exact frequency (49, 50). Similarly, the relationship of 9p21 genetic events to tumour grade, stage and recurrence has not been clear. HD has been reported to be associated with larger tumour size and reduced recurrence-free interval (51) but some studies have found no relationship with recurrence (52). Recently, we carried out a comprehensive screen of all known mechanisms of inactivation of p16/p14^{ARF} using microdissected tumour samples to avoid possible underscoring of homozygous deletions due to contaminating normal cellular DNA (53). This included an assessment of gene dosage by quantitative real-time PCR for exon 2 (shared by p16 and p14^{ARF}) and exon 1β (p14^{ARF} only), LOH, promoter hypermethylation, mutation status and p16 protein expression. HD of exons 2 and 1β was detected in 35% and 22% of tumours, respectively. As reported in other studies, co-deletion was most common, but exon 2-specific HD was also detected. In tumours without HD, p16 promoter hypermethylation was detected in only 1 case and hypermethylation of the p14^{ARF} promoter and mutations in the *CDKN2A* coding sequence were not observed, confirming several previous studies. Both HD of exon 2 and LOH of 9p were associated with higher tumour stage. The use of pure tumour DNA allowed genomic underrepresentation to be measured accurately. Exon 2 was

under-represented in 43% of cases and exon 1β in 46% of cases. Interestingly, it was found that a reduction in gene dosage of exon 1 and exon 2 was only accompanied by LOH in 30% or 50% of cases respectively. This implies that many of these tumours were at least triploid and retained two parental alleles for *CDKN2A* with loss of at least one copy of the gene. This suggests that *CDKN2A* may be haploinsufficient in the urothelium. Underrepresentation of the chromosome 9 centromere as measured by *in situ* hybridisation on tissue sections was also reported in a previous study in 5/18 cases with no LOH (54). Knockout mouse studies (reviewed in (55)) and *in vitro* experiments on mouse cells (56) have also suggested that haploinsufficiency of p16 and/or p14ARF is significant. In our recent study, either LOH and/or underrepresentation was found in 78% of tumours. HD of exon 2 and LOH anywhere on chromosome 9 were associated with tumour invasion (53). This association of HD and LOH with higher tumour stage may indicate that loss of one copy by physical underrepresentation may be sufficient for development of a low grade/stage tumour but that complete loss of function can confer an additional advantage. These results now require independent confirmation but if correct this implicates the 9p21 genes in the vast majority of bladder tumours.

p16 is a negative regulator of the Rb pathway (Figure 5) and loss of p16 represents an alternative to Rb loss as a mechanism to inactivate the pathway and dysregulate the G1/S checkpoint. In tumours where Rb is inactivated, it has been shown that p16 expression is upregulated and that an inverse relationship between Rb and p16 expression exists (57, 58). Thus, abnormally high levels of p16 are indicative of RB loss and may be biologically equivalent to p16 loss and *vice versa*. Several studies have examined the relationship of abnormal p16 expression as detected by immunohistochemistry (no detectable expression or high homogeneous staining) to a range of clinical parameters. A recent study showed that abnormal expression of p16 (and of Rb) was related to disease progression following cystectomy (59). An association of altered p16 staining (loss of expression) with reduced progression-free survival has been reported for Ta and T1 tumours (60). This association of compete inactivation of the locus with more aggressive disease again raises the possibility that the underrepresentation commonly seen in low grade/stage tumours with an accompanying partial loss of expression may lead to an abnormal phenotype.
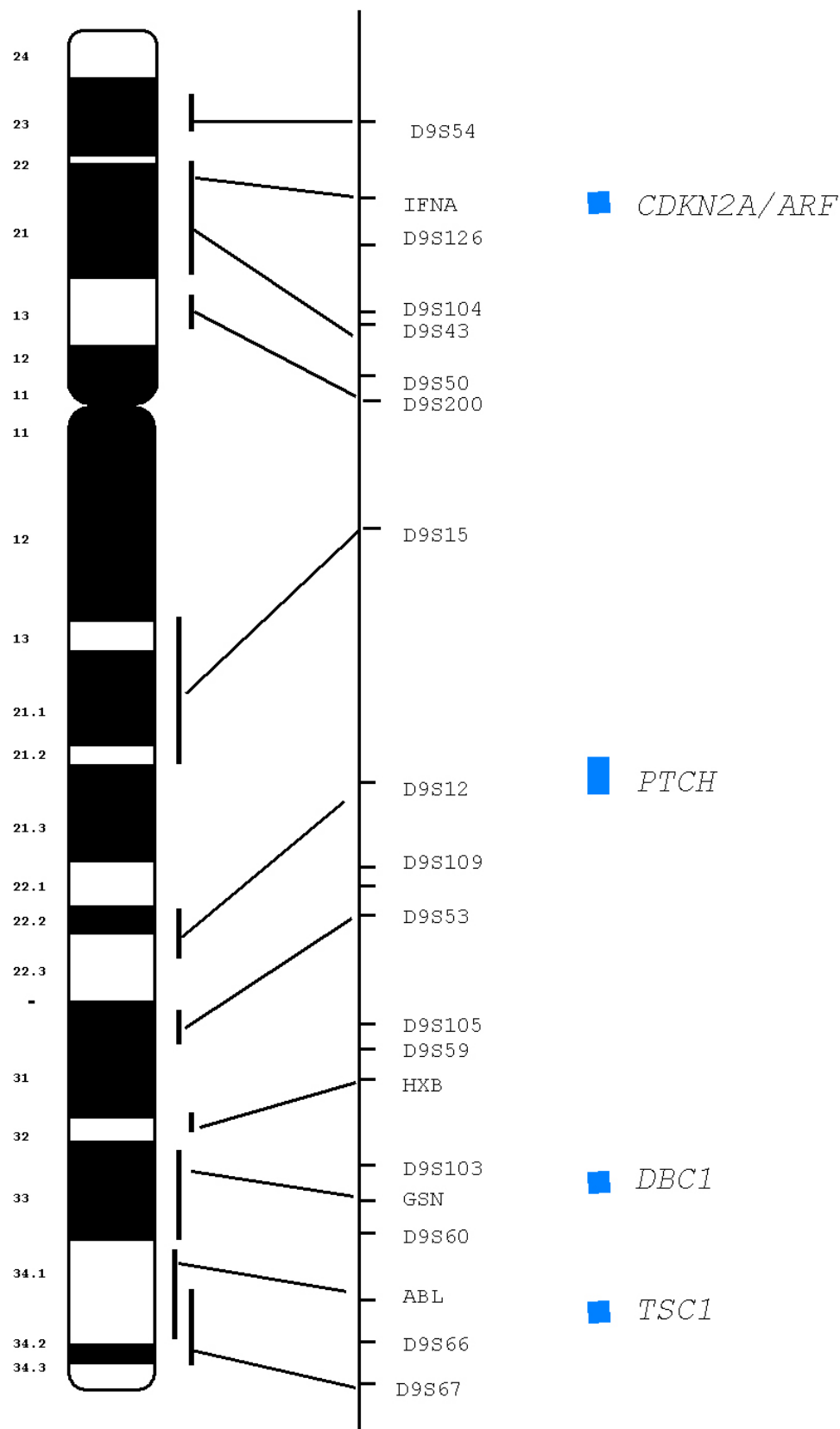
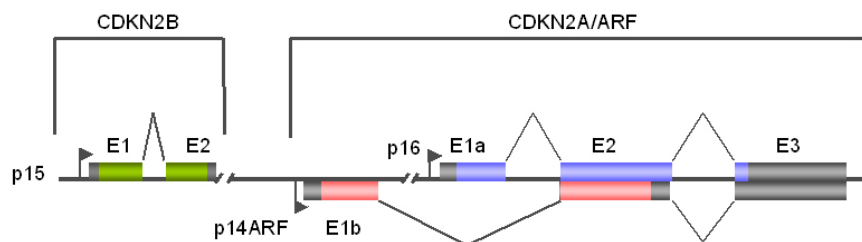**Figure 3.** Candidate tumour suppressor genes on chromosome 9.

Figure 4. Genomic organisation of the 9p21 loci *CDKN2B* and *CDKN2A/ARF* encoding p15, p16 and p14^{ARF}. p16 and p14ARF share exons 2 and 3 but have distinct exons 1. Transcripts read in different reading frames generate two different protein products.
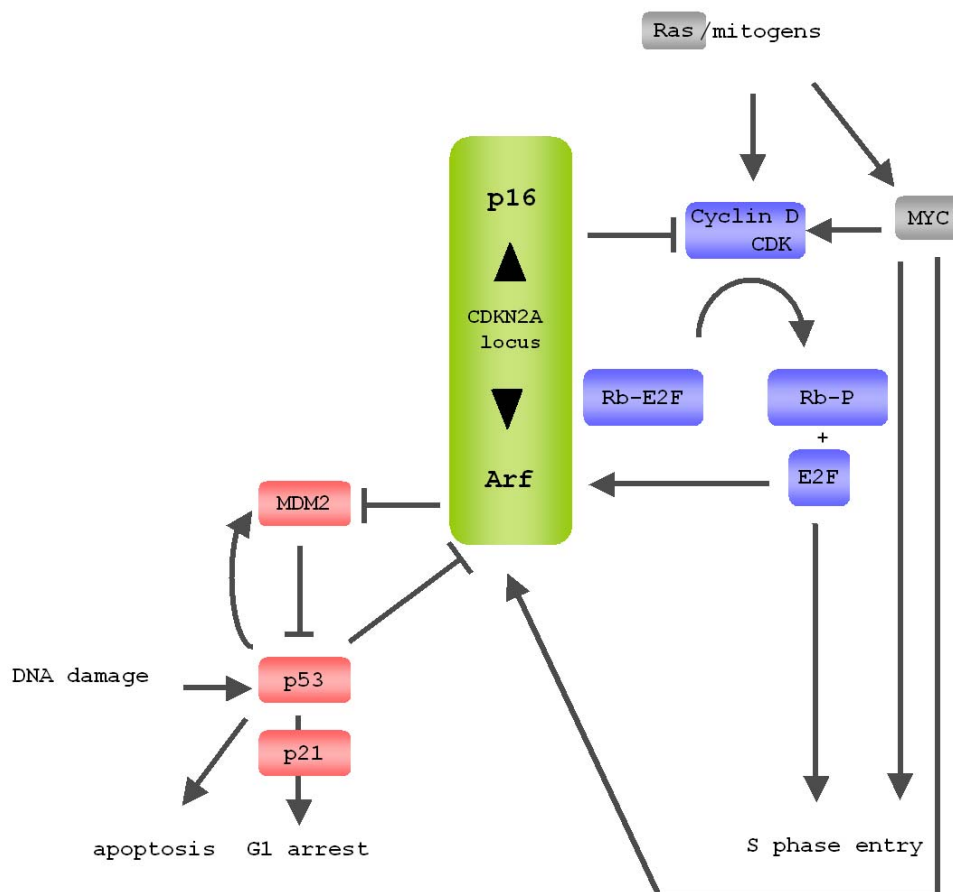

Figure 5. Rb and p53 pathways. Much of the information summarised has come from experiments on rodent cells and although much has been confirmed in human systems, the roles of p16 and p14^{ARF} in human cells may differ. Only key interactions are shown. There are many known positive and negative feedback loops that presumably allow exquisite control of these pathways in multiple cell types and situations. The *CDKN2A* locus encodes p16 and p14^{ARF} that act as negative regulators of the Rb and p53 pathways respectively. This interrelated signalling network regulates tumour suppression via 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 (not shown). This allows formation of cyclin E-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 a range of post-translational modifications that both increase its half-life and render it more active as a transcription factor. p21 expression is induced and this leads to cell cycle arrest via inhibition of cyclinE-CDK2 or apoptosis, depending on cellular context. MDM2 is a ubiquitin ligase responsible for inactivation of p53. In turn p53 regulates MDM2 expression providing a negative feedback loop. The p53 and Rb pathways are connected by p14^{ARF}, which sequesters MDM2 in the nucleus. Over-expression of E2Fs and oncogenes such as MYC can both result in p53-triggered cell cycle arrest via p14^{ARF}. All proteins shown are altered in function or expression level in UCC.
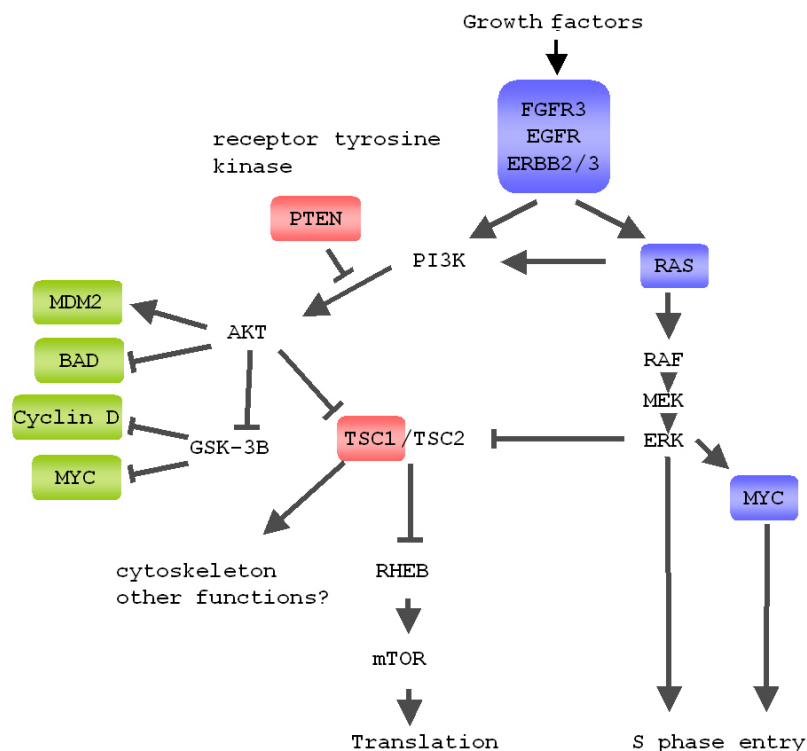
Figure 6. Oncogenic signalling via the MAPK and PI3-kinase pathways. Growth factor mediated signalling or mutational activation of Ras oncogenes can activate both of these pathways. Signalling via the RAS/RAF/MEK/ERK cascade leads to phosphorylation of many substrates that can have multiple cellular effects depending on the intensity and duration of signalling. In many situations proliferation is induced. Activated receptor tyrosine kinases bind p85, the regulatory subunit of PI3-kinase and recruit the enzyme to the membrane where it phosphorylates phosphatidyinositol–4,5-bisphosphate (PIP2) to generate PIP3 which in turn recruits ATK and PDK1 (not shown) to the membrane where AKT is activated by phosphorylation to regulate a wide range of target proteins (not all shown). Amongst these are cyclin D1 and MDM2 which are upregulated either directly or indirectly, resulting in a positive stimulus via the p53 or Rb pathways respectively. AKT also phosphorylates and inactivates tuberin the *TSC2* gene product, leading to activation of the mTOR pathway which controls protein synthesis. The *TSC1* product hamartin forms an active complex with tuberin and loss of function of either protein leads to dysregulated mTOR signalling. MYC expression is induced as a consequence of both by ERK and AKT signalling. Coloured shading indicates proteins altered in function or expression level in UCC.

5.2. *PTCH*

A minimum region of deletion has been mapped via LOH analysis to 9q22 (27). This contains the Gorlin syndrome gene *PTCH*. Mutations of the gene are infrequent in UCC (40, 61, 62) but reduction in mRNA expression is common and it has been suggested that this gene may be haploinsufficient in the urothelium (31). Further evidence for the relevance of this gene to bladder cancer development has come from the finding that *Ptch*+/- heterozygous mice develop preneoplastic and neoplastic changes in the bladder earlier than wildtype mice in response to treatment with the carcinogen *N*-butyl-*N*-(4-hydroxybutyl) nitrosamine (63).

5.3. *DBC1*

At 9q33, a novel gene, *DBC1,* is the only gene within the common region of deletion. Homozygous deletion has been found in a few tumours (41, 42) and although there is no evidence of mutational inactivation, there is common transcriptional silencing by hypermethylation of the promoter (32, 64). The function of *DBC1* is not yet clear but it has been shown that ectopic

expression can induce a non-apoptotic form of cell death (65) and in cells that survive, there is a delay in the G1 phase of the cell cycle (66).

5.4. *TSC1*

A third candidate bladder TSG on 9q is the tuberous sclerosis gene *TSC1* at 9q34. Germline mutation of *TSC1* or of a second gene *TSC2* (16p13) is associated with the familial hamartoma syndrome tuberous sclerosis complex (TSC). In bladder tumours, mutations of *TSC1* are found in 10-13% of cases (43-45). Interestingly some of these mutations are in tumours without 9q34 LOH, indicating as for the other chromosome 9 TSGs the possible role of haploinsufficiency (45). The effect of *TSC1* loss on bladder tumour phenotype is not yet clear but one report indicates an association of 9q34 LOH with recurrence (67).

The *TSC1* gene product hamartin acts in complex with the *TSC2* gene product tuberin in the PI3-kinase pathway to negatively regulate mTOR, a central molecule in the control of protein synthesis and cell growth (68) (Figure 6). Currently it is not clear why patients with TSC

develop only benign hamartomas and only very rarely develop malignant tumours. Understanding of the signalling processes affected by TSC1 and TSC2 suggests that this may be explained by the induction of a negative regulatory feedback loop to suppress phosphorylation of AKT in *TSC1* or *TSC2* null cells (for review see (69)). In accord with this hypothesis, no mutations have been reported in these two genes in any human cancers apart from bladder. Why the urothelium is an exception is not clear and careful searching for possible tissue specific differences in PI3-kinase pathway signalling or other TSC1/2-related functions is now needed. *Tsc1* and *Tsc2* knockout mice (+/-) survive for long periods and develop several of the features of TSC. Neither these nor TSC patients appear to have increased risk of bladder cancer.

6. *TP53* AND *RB1*

Physical deletion and LOH of 17p are common findings in bladder tumours (7, 21) and this is linked to mutation of the *TP53* gene (70-74). Deletions of chromosome 13 have also been described and the increased risk of bladder cancer in retinoblastoma family members (75, 76) together with the finding of LOH in the region of the *RB1* gene (77) identified this as a potential bladder tumour suppressor gene. This is a large gene and has not been systematically screened for mutations in bladder tumours. Nevertheless, it has been shown that 13q LOH is almost always accompanied by loss of expression of the Rb protein (78)

6.1. p53, Rb and their pathways

The interconnecting pathways controlled by p53 and Rb regulate cell cycle progression and responses to stress, processes that are almost universally deregulated in malignant cells (for review see (79)) (Figure 5). p53 plays a key role in determining cellular response to various stress signals. In the absence of stress stimuli, p53 protein levels are low but when activated, protein levels rise and transcription of a wide range of genes is activated. p53 activation induces apoptosis in some circumstances and cell cycle arrest in others depending on the cell type and the nature of the stimulus. Mutation of *TP53* is found in many muscle invasive bladder cancers (70-73, 80). As many mutations increase the half life of the protein, detection of high levels of protein by immunohistochemistry has been used as a surrogate marker for mutation (81). However, it should be noted that a significant number of mutations do not lead to protein stabilisation and mutation screening represents the best measure of p53 status. p53 accumulation has been associated with adverse prognosis in all types of UCC (82-85) and it has been suggested that this might be used as a prognostic marker in the clinic. Similarly, expression of the cyclin dependent kinase p21 which acts downstream of p53, has a reported association with better prognosis (86). It has also been reported that patients with tumours that retain p21 expression, even with p53 mutation, have outcomes similar to those with wildtype p53 (86, 87). However, not all studies have confirmed these findings and a recent metaanalysis of more than 3000 tumours found only a weak association between p53 positivity by immunohistochemistry and poor prognosis

(88). In the future, it will be important to measure not merely the status of p53 but the integrity of the p53 response, which may be disrupted by a range of mechanisms in human cancers.

Rb acts in a pathway that regulates progression from G1 to S phase of the cell cycle (Figure 5). It binds to members of the E2F transcription factor family and this complex recruits histone deacetylases to E2F responsive promoters. Cdk-mediated phosphorylation of Rb prevents association with E2F and enables E2F-mediated gene expression and progression into S phase. Some homozygous deletions of *RB1*, LOH of 13q14 and loss of Rb protein expression have been detected in UCC. The frequency of loss of Rb as for inactivation of p53 is higher in tumours of high grade and stage (77, 78, 89, 90).

As indicated above, the locus encoding p16 and p14[ARF] is commonly deleted in UCC of all grades and stages. These proteins interact with and link the Rb and p53 pathways (Figure 4) and due to the multiple regulatory feedback mechanisms that operate in these pathways (91), inactivation of both of these together is likely to provide further freedom from the G1 checkpoint than conferred by either p53 or Rb inactivation alone. Thus 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 (59, 92). To date, an assessment of all genes known to be involved in the G1 checkpoint has not been carried out on a single patient series but such an analysis may achieve even greater predictive power.

Cyclin D1, which binds to CDKs resulting in phosphorylation of Rb and release of E2Fs required for cell cycle progression, is over-expressed as the result of DNA amplification in some bladder tumours including some superficial tumours. Over-expression of cyclin D1 represents another possible way to inactivate Rb and override the G1 checkpoint and this has been associated with low grade tumours with papillary architecture (93). The frequency of amplification is insufficient to explain all cases with observed over-expression. Interestingly, cyclin D1 is a possible target of the Wnt/β–catenin pathway and recently it was reported that some high grade bladder tumours (3/59 studied) had mutations in β-catenin (94). This represents an alternative mechanism for cyclin D1 activation in these tumours and appears to be associated with a more aggressive phenotype than the over-expression associated with gene amplification, found in low grade superficial tumours.

7. PTEN

PTEN (**p**hosphatase and **ten**sin homologue deleted on chromosome **ten**) maps to 10q23, a region of common LOH in UCC of high grade and stage (95-97). PTEN has both lipid and protein phosphatase activity, a key substrate being the signalling lipid PtIns(3,4,5)P$_3$ a major

Table 4. Genes reported to show promoter methylation in bladder cancer

Gene	Location	Frequency	Association	References
RASSF1A	3p21	35-79%	High stage, upper tract tumours	106, 108, 147-150
DAPK	9q34.1	6-58%	Early recurrence	108, 147, 150-152
CDH1 (E-cadherin)	16q22.1	36-84%	Invasion, shortened survival	109, 150-152
FHIT	3p21	11-16%	Shortened survival	150, 153-155
APC		35-69%	Grade, stage	106, 150, 156
CDKN2A (p16)	9p21	7-60%		49, 51, 53, 106, 147, 150, 151, 157-160
CDKN2A (p14^{ARF})	9p21	0-56%	Stage, tumour multicentricity, larger tumour size, relapse	49, 53, 106, 147, 157, 158
CDKN2B (p15)	9p21	0-13%		151, 161
RUNX3	1p36	50-73%	Stage, progression, recurrence of superficial	162, 163
SFRP genes	SFRP1, 8p12-p11 SFRP2, 4q31.3 SFRP4, 7p14.1 SFRP5, 10q24.1	18% 52% 9% 37%		164
WWOX	16q23.3	29%		154
HPP1	2q32.3	35%	Stage	163
GREM1	15q13-q15	50%	Stage	163
MLH1	3p21.3	13-25%		152, 158
TERT	5p15.3	25%	Grade	108
BCL2	18q21.3	52%	Stage, grade	108

product of PI3-kinase which is activated by various tyrosine kinase receptors (Figure 6). Thus PTEN is a negative regulator of this signalling pathway, which affects cell phenotype in various ways including effects on proliferation, apoptosis and cell migration (98). Heterozygous knockout mice (*Pten* +/-) show widespread proliferative changes, suggesting that loss of one allele 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 homozygous deletion in some bladder tumour cell lines (99-101). However, most bladder tumours with 10q LOH do not show demonstrable inactivation of the retained allele indicating the possibility that reduction in PTEN expression may be sufficient to contribute to tumour development. To date, a large bladder tumour series has not been assessed for levels of PTEN expression.

It has been reported that PTEN re-expression in bladder tumour cells that lack functional PTEN suppresses tumour growth *in vivo* (102, 103). Re-expression experiments also show an effect on tumour cell chemotaxis and anchorage independent growth (104). Using PTEN mutants that lack either lipid phosphatase activity, protein phosphatase activity or both, it was shown that the lipid phosphatase activity of PTEN is required to inhibit anchorage independent growth under low serum conditions and that the protein phosphatase activity is required to prevent cell chemotaxis and tumour cell invasion in an organotypic assay.

Loss of PTEN leads to PI3-kinase pathway activation and is associated with high levels of phosphorylated AKT. As discussed above, the *TSC1* product hamartin also acts in the PI3-kinase pathway (Figure 6), 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 (105), 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 alternative mechanisms of activation.

8. GENES INACTIVATED BY PROMOTER METHYLATION

Some of the genes already mentioned can show promoter methylation as one possible mechanism of transcriptional silencing in cancer. These include *RB1* and *DBC1*. Although methylation of the *RB1* promoter has been identified in other cancers, this not yet been reported in bladder tumours. However, only a limited number of samples have been studied to date (106). Methylation of the *DBC1* promoter is relatively common (32, 64, 107).

Promoter methylation of many other genes has been found in bladder tumours. Some of these are mapped within regions that show LOH (Table 4). *RASSF1A,* a candidate tumour suppressor gene mapping to 3p21, a region that commonly shows LOH in bladder cancer, has been found to be methylated in many bladder tumours and this is associated with high tumour stage. It should be noted however that this gene promoter and those of some other genes which commonly show methylation are also reported to be methylated in normal bladder tissue samples, albeit at a lower frequency (108, 109). Currently, the range of frequencies reported for methylation of specific genes is wide and it is not yet clear whether such inter-study differences represent technical difficulties or whether there are significant differences in the study populations. As PCR-based methylation analysis including real-time quantitative analysis is now becoming routine, these differences may soon be resolved. Clearly epigenetic changes are common in bladder cancer and it will be very important to confirm initial findings and integrate this information with genomic information and clinical data in the future.

9. CANDIDATE TUMOUR SUPPRESSOR REGIONS

Both LOH and CGH analyses have identified regions of the genome that show copy number or allelic loss in bladder tumours (Tables 1 and 2). For most of these, the target genes have not yet been identified. A key region of loss is the short arm of chromosome 8. 8p LOH shows significant association with high tumour grade and stage (110-114). Several common regions of deletion on 8p have been mapped

by LOH analysis in UCC. These are a telomeric region mapped at 8p23 (115), a region at 8p22 (112, 114) and at least 3 small, non-coincident regions on 8p11-p12 (110, 111). The region of LOH on 8p22 overlaps a region mapped in oesophageal cancer within which a candidate gene *LZTS1* (originally called FEZ1) was identified (116). This gene has been screened for mutations but only one possible mutation was found in a panel of 54 bladder tumours and 32 cell lines (117). A second candidate gene mapped to the 8p22 region of deletion, *DBC2* (deleted in breast cancer 2) (118), also known as *RHOBTB2* was also screened in the same study and only a single mutation was identified in a tumour sample. This was within a conserved region of the gene suggesting that it might be biologically significant. The frequency of mutation of these candidates is therefore too low to implicate them as the major target genes on 8p. However, the possibility remains that either or both of these genes may be more commonly inactivated by a mechanism other than mutation in UCC.

Similarly, TRAIL receptor 2 (TRAIL-R2) also known as *TNFRSF10B* (Tumour necrosis factor receptor super-family 10b) or KILLER/DR5, a member of the tumour necrosis factor receptor (TNFR) family, is a promising candidate tumour suppressor gene at 8p21-22. Mutations in this gene have been identified in non-small cell lung cancer, head and neck cancer, breast cancer and non-Hodgkin's lymphoma. Screening of 26 UCC tissues and 15 bladder tumour cell lines identified only one novel protein truncating mutation in a bladder cancer cell line (119). Again, this suggests that if TRAIL-R2 is the target of LOH events in these cancers, inactivation of the remaining allele is by a mechanism other than mutation.

Approximately 40% of UCCs have 11p LOH and again no good candidate gene has been identified. One suggested candidate is *CDKN1C* (p57KIP2) at 11p15.5. It has been reported that many advanced UCC show downregulation of CDKN1C expression and promoter methylation has been found in several tumour cell lines (120). Since loss of expression did not correlate well with 11p LOH, further studies are needed to establish the role of this gene in UCC development.

Loss of 3p has been reported in ~48% of UCC and is associated with invasion (121, 122). LOH has been found in 3 regions, 3p12-14, 3p21.3-22 and 3p24.2-25 (123). Deletions in some cases include the *FHIT* locus, which commonly shows promoter methylation (Table 4). Candidate genes in the other 3p regions of deletion have not been assessed.

There are many other regions of LOH and/or copy number loss described at lower frequency in UCC (Table 1 and 2). These may represent critical events in specific sub-groups of tumours. To date however, candidate genes in these regions have not been assessed.

10. THERAPEUTIC APPROACHES TO TARGET TUMOURS WITH SPECIFIC TUMOUR SUPPRESSOR ALTERATIONS

Knowledge of the genetic pathways involved in tumour development provides clues to the critical or rate-

limiting events that may be required for tumour maintenance or survival and may allow rational targeted therapies to be developed. Many targets currently generating interest in the scientific and pharmaceutical communities are oncogenes that may be inhibited by small molecules or antibody-based therapies. Strategies to replace tumour suppressor gene function are more difficult to devise. Several groups have attempted to use replacement gene therapy to deliver wildtype p53 or Rb proteins to cultured tumour cells or tumours maintained as xenografts in mice (124, 125). An adenoviral vector expressing p53 has been tested intravesically in a phase I study in patients and a high degree of tolerance was reported (126). However, problems have been identified with infectivity of some bladder tumour cells due to loss of expression of the coxsackie adenovirus receptor (127) and with penetration of virus to deeper layers of tumours (128). A recent study has reported the use of a transduction enhancing agent (Big CHAP) to overcome the latter problem and enhance transgene expression following intravesical adenovirus instillation (129). This may allow successful adenovirus-mediated gene replacement therapies for bladder-confined disease in the future.

An alternative approach has been to exploit the molecular phenotype of tumours to generate specific virus tropisms. Several such viruses, termed oncolytic adenoviruses, have now been generated. The prototype was the replication defective adenovirus ONYX-015 which has an E1B-55K deletion. Theoretically this should allow virus replication only in cells with defective p53 (130). Similar viruses with deletions in the E1A region have been designed to target Rb deficient cells e.g. (131). No oncolytic viruses have yet been tested in bladder cancer but it can be envisaged that in combination with an agent to enhance viral transduction, these may by useful agents for localised disease with mutations in appropriate genes.

A recent approach for replacement of tumour suppressor gene activity is the development of small molecules that either induce conformational changes in the target protein such that wildtype function is restored or that interfere with other proteins that cause downregulation of tumour suppressor proteins such as MDM2 (132-134). As yet these have not been tested in UCC but the wide range of inactivating point mutations found in *TP53* make this an attractive approach for therapy of advanced disease.

11. SUMMARY AND PERSPECTIVE

A wealth of information on TSG loci involved in bladder cancer now exists. Acquisition of this information has been driven largely by the hypothesis that knowledge of the heritable events involved in tumour development will provide valuable insights into tumour phenotype and will have application in the clinic. To date no routine applications of this information have been developed. Although many potential markers have been identified, clinical associations have not been confirmed in large patient cohorts. In the case of *TP53* and *RB1*, both genes whose mutation is clearly associated with high tumour grade and stage, it has been difficult to use these in a

meaningful way in the clinic. It can be argued that this is due to an incomplete picture resulting from failure to identify all tumours with similar phenotype. Larger panels of markers that cover all possible molecular mechanisms that generate the same clinical phenotype may be needed before sufficient predictive power can be achieved. If our aim is to use tumour molecular biology as a means to improve clinical management, it is desirable that we identify those molecular changes that have most impact on tumour cell phenotype. This requires an understanding of the key features of tumour cells that are likely to influence their behaviour. We now know the identity of many of the key tumour suppressor genes and it is essential that careful functional studies are carried out to elucidate the relationship of these to cellular behaviour. In this context, both mouse models and *in vitro* studies using human urothelial cells are likely to be pivotal.

12. ACKNOWLEDGEMENTS

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Abbreviations: LOH: loss of heterozygosity; UCC: urothelial cell carcinoma; TSG(s): tumour suppressor gene(s); PCR: polymerase chain reaction; HD: homozygous deletion; TSC: tuberous sclerosis complex

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