Molecular biology of bladder cancer

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Bladder cancer is a major cause of health expenses and it presents formidable clinical challenges. Two types of tumors have been identified, papillary and non-papillary. The former are mainly characterized by FGFR3 and chromosome 9 alterations and a low frequency of Tp53 alterations. The latter are characterized by a high frequency of alterations in genes in the p53 and Rb pathways. Chromosome 9 alterations, specially in 9q, are crucial to bladder cancer development and occur in both types of tumors. Progression of some superficial tumors (mainly TaG3 and T1G3) to high-grade, invasive, carcinomas provides evidence of some overlap between the two pathways. Distinct gene expression profiles have been identified in superficial and invasive tumors. The stage is now ready for the clinical application of this knowledge.

Key words Bladder • FGFR3 • Genetic alterations • p55 Rb • Urothelial carcinoma


INTRODUCTION

Bladder cancer is a major health burden in the Western world. In 2002, approximately 159,000 new bladder cancer cases were diagnosed in Europe, the male:female ratio being 3.7:1. In the same year, approximately 51,000 patients died from this disease. In Spain, it is the 5th leading cause of cancer-related death in men and the 15th in women. Bladder cancer generates the highest medical cost per patient and it is the fifth most expensive cancer in terms of total medical care expenditures.

The two best established lifestyle/environmental risk factors for bladder cancer are smoking and occupational exposure to aromatic amines (i.e. dye, textile, leather, chemical and rubber industries). Therefore, this tumor is a largely preventable disease.

BLADDER TUMORS

Approximately 95% of malignant bladder tumors are urothelial cell carcinomas (UGC) which can be classified as papillary (most common type, tend to grow slowly towards the lumen), solid (less frequent, infiltrate the bladder wall and are more aggressive) or Carcinoma In Situ (CIS, a very aggressive kind of cancer that involves only the inner lining of the bladder).

Staging and grading

The bladder is a hollow and distensible organ that sits on the pelvic floor and collects urine from the kidneys. A scheme of the bladder wall is represented in figure 1.

Tumors are classified according to depth of invasion into superficial (Ta and T1) and invasive (T2, T3, T4). pTa tumors are defined as those which have an exophytic «fingerlike» growth and do not grow beyond the urothelium; pT1 tumors invade the lamina propria but not the muscularis propria; pT2 tumors as those invading the muscularis propria; pT3 tumors as those invading perivesical tissue; and pT4 tumors as those invading other organ structures.

Grading is used to evaluate the cytological and/or growth pattern characteristics of the tumor and it is an important predictor of its biologic potential. 

1. Flat and papillary hyperplasia. Flat hyperplasia consists of a markedly thickened mucosa without cytologic atypia. Papillary hyperplasia is characterized by urothelium of variable thickness exhibiting a slightly undulating growth.
2. Flat lesions with atypia can be classified as reactive atypia, dysplasia or CIS. Dysplasia indicates cytologic and architectural abnormalities.

3. Papillary neoplasms can be classified as papilloma, papillary neoplasm of low malignancy potential (PUNLMP), low grade papillary carcinoma and high grade papillary carcinoma. In papilloma, normal appearing urothelium lines papillary fronds. PUNLMP refers to thickened urothelium in the absence of cytologic features of malignancy. Low grade papillary carcinoma exhibits an overall orderly appearance and has minimal anomalies in architecture and/or cytologic features. High grade papillary carcinoma has a disordered appearance with marked architectural and cytologic abnormalities.

**Natural history of bladder tumors: two pathways, clonal origin**

UCC is a heterogeneous disease with a variable natural history. Low-grade Ta tumors have a very low progression rate and rarely present a threat to the patient. At the other extreme, high-grade tumors imply a high risk of progression and cancer death.

**Two pathways**

It has been proposed that there may be at least two separate pathways leading to bladder cancer, papillary and nonpapillary. Papillary lesions arise in hyperplastic urothelium whereas invasive tumors arise from dysplastic urothelium. The overlap between both pathways is reflected by progression of superficial papillary tumors to high grade invasive carcinomas in some cases. Papillary tumors are commonly associated with alterations in genes involved in the RAF/MEK/ERK pathway (i.e. FGFR3)\(^5,6\) and in PIK3CA\(^7\). By contrast, non-papillary invasive tumors are commonly associated with p53 and pRb pathway alterations (fig. 2).

**Clonal origin**

The development of multiple tumors in either a synchronous or metachronous manner in the same patient is a common characteristic of UCC. Two theories have been proposed to account for these findings: tumors may be monoclonal in origin (multifocal tumors evolving from a single transformed cell) or they may be oligoclonal (a change in the urothelium giving rise to multiple clones of initiated cells subsequently evolving into independent tumors). Current evidence supports the notion that most tumors are monoclonal but up to 50% of tumors may indeed be oligoclonal\(^8,9\).

**Genetic alterations in bladder cancer**

For many years, it has been clear that UCC represents a complex disease and that it is a paradigm of tumor progression. The current status of knowledge on the molecular biology of bladder cancer is presented, pointing out the differences between papillary and invasive bladder tumors.

**MOLECULAR GENETICS OF PAPILLARY BLADDER TUMORS**

**Fibroblast growth factor receptor 3 (FGFR3)**

FGFRs (1-4) regulate proliferation, differentiation, angiogenesis and embryonic development. They display distinct tissue expression, ligand specificity, signal
pathway activation, and biological effects. FGFR mRNAs are commonly subject to alternative splicing. There are two major isoforms of FGFR3 transcripts that are generated from a mutually exclusive splicing event in which the second half of the third Ig-like domain is encoded by either the 151 bp of exon 8 (FGFR3b, predominant in epithelia) or the 145 bp of exon 9 (FGFR3c, predominant in mesenchymal cells). Inherited skeletal dysplasias have been linked to activating germline point mutations in \textit{FGFR1}, \textit{2} and \textit{3}. Germline point mutations in \textit{FGFR3} cause the short limb syndromes of achondroplasia, hypochondroplasia and thanatophoric dysplasia. Mutations lead to constitutive receptor activation; increasing activity is associated with increasing severity of the disease\textsuperscript{10,11}. Activating \textit{FGFR3} mutations occur in 50-60\% of UCC and are associated with papillary, low grade, superficial tumors\textsuperscript{5}. Somatic mutations are restricted to a few codons, essentially the same that are mutated in the germine in short limb syndromes. Mutations in the extracellular domain (i.e. codons 248, 249) or within the transmembrane domain and its vicinity (i.e. codons 372, 375, 382, 393) lead to receptor dimerization in the absence of ligand; mutations in the intracellular domain (codon 652) confer constitutive kinase activity. Mutations are associated with an increased risk of recurrence and a lower risk of progression among patients with superficial tumors, indicating that they are a marker of tumors with good prognosis\textsuperscript{12}.

\textbf{Ras genes}

\textit{H-ras}, \textit{K-ras}, and \textit{N-ras} code for monomeric GTPases that can activate the RAF/MEK/ERK and PI3K/AKT/PTEN cascades. Oncogenic Ras mutations make the GTPase insensitive to the action of activating proteins (GAPs) and lock it in the GTP-bound, active state. In UCC, point mutations have been identified in codons 12, 13 and 61 in \textit{H-ras} and in \textit{N-ras} at an overall frequency of 10-20\%. \textit{FGFR3} and \textit{Ras} mutations are mutually exclusive, possibly because they both activate the MAPK pathway. \textit{Ras} mutations do not seem to be associated with stage or grade\textsuperscript{13}. Amplification of the gene coding for Raf1, a kinase involved in Ras protein signalling, has been associated to grade, stage and poor survival and overexpression has been associated to grade, but not to stage\textsuperscript{14,15}.

\textbf{PIK3CA and TSC1}

The phosphatidylinositol-3-kinase (PI3K) pathway is crucial to many aspects of cell growth and survival. Class IA proteins are most important in proliferation and tumorigenesis; they are constituted by a heterodimer of p110, the catalytic subunit, and an adapter subunit (p85, p50 or p55). \textit{PIK3CA}, which encodes p110\(\alpha\), maps to 3q26, a region amplified in UCC. \textit{PIK3CA} mutations have been reported in 20\% of tumors, in association with low stage and grade tumors and with \textit{FGFR3} mutations\textsuperscript{7}. There is some evidence that UCC can also overexpress PI3K in comparison with adjacent normal epithelium. TSC1 (9q34) encodes hamartin, a GAP that negatively regulates cell cycle progression. Its action is blocked by AKT and activation of the PI3K pathway. Inactivating TSC1 mutations are found in 12\% of UCC and LOH at 9q34 occurs in 32\% of cases\textsuperscript{16,17}. Stage and grade do not seem associated with these alterations.
MOLECULAR GENETICS OF INVASIVE TUMORS

Virtually all human tumors deregulate either the Rb or p53 pathway or both and this is also the case for invasive UCC.

Rb pathway alterations

The Rb pathway is responsible for regulating the passage from G1 to S phase. Phosphorylation of pRb by CDK4/6 and CDK2 dissociates the pRb-repressor complex, leading to the release of bound E2F from pRb. «Free» E2F is then active and drives the transcription of S-phase genes encoding for proteins required for G1 to S phase transition and DNA replication.

Mutations of the Rb1 gene have been described in UCC but the studies are not extensive due to the large gene size. There is controversy about the prognostic value of pRb expression in tumor tissues. p16, encoded by the INK4A/ARF locus, is a CDK4/6 inhibitor which blocks the phosphorylation of pRb. The INK4A/ARF locus plays a central role in tumor suppression and it is inactivated in approximately 50% of human cancers. mRNA levels of p16INK4A and p14ARF are undetectable in normal urothelium and increase with stage and grade. At the protein level, loss of p16 has been associated to minimally invasive bladder cancer. p16INK4A and p14ARF methylation have been proposed as biomarkers of stage, clinical outcome, and prognosis.

E2F1 expression has been correlated with proliferation in UCC, having a growth promoting effect. The E2F3 locus is amplified in invasive tumors and its product is overexpressed in 33% of UCC.

Fig. 3. Receptor tyrosine kinases are activated by ligand and, through the recruitment of adaptor proteins containing SH2 domains, signal to Ras and the ERK/MAPK pathway which is mainly involved in proliferation. In the case of FGFR3, specific adaptors such as Frs-2 have been implicated. In addition, they can signal to the PI3K pathway thus leading to survival and inhibition of apoptosis. The Akt kinase mediates these effects by modulating the activity of several proteins such as NF-κB, GSK-3β, and mTOR. PI3K is also involved in the regulation of cell motility and cytoskeletal remodelling.
Cyclin D1 overexpression occurs frequently in UCC and has been proposed to be associated with the growth of low-grade papillary tumors.

### p53 pathway alterations

The p53 pathway plays a major role in the response to DNA damage, oncogenic stress, and other types of cellular stress. p53 induces G1 arrest by upregulating p21, G2 arrest by upregulating GADD45, 14-3-3σ and p21, and apoptosis by upregulating genes such as Bax, NOXA, and PUMA. In addition, it can repress genes such as c-myc to promote G1 arrest and cyclin B1 to promote G2 arrest.

The Tp53 gene is frequently altered in UCC: 270 different mutations have been registered in the IARC database up to October 2006, of which 262 (97%) are in exons 4 to 9. The most common mutations are missense (72.5%), 12.2% and 5.55% being nonsense and silent, respectively. The main hotspots are codons 285, 248, 280, 175 and 213. Tp53 mutations are significantly more frequent among high grade and stage tumors; their prevalence ranging from 14-52% depending on the T and G subgroup considered.

Overall, Tp53 mutations and p53 nuclear overexpression are uncommon in TaG1/G2 tumors and are frequent in high grade/invasive tumors. PTEN, a p53 target gene and regulator, is gained/amplified in approximately 9% of high grade tumors.

p63 and p73 are members of the p53 family. p63 maps to 5q27-29, a region that is amplified in UCC; this locus encodes multiple proteins resulting from alternative splicing that transactivate p53 responsive elements or act as dominant negatives towards p53 and p73. p63 is lost in most invasive tumors. A decrease of the levels of p75 has also been associated to bladder cancer stage.

Two additional important genes in the p53 pathway are Aurora A kinase (STK6) and p21. Overexpression of Aurora A (20q15), often associated with gene amplification, leads to increased degradation of p53, causing down-regulation of checkpoint-response pathways and facilitating oncogenic transformation. Expression of p21 is reduced in muscle invasive tumors compared to non-invasive tumors.

A few studies have combined the analysis of p53, p16, p21 and pRb expression in bladder tumors and described that they act in cooperative or synergistic ways to promote progression: p55/p16, p55/p21, p55/pRb or p55/p21/pRb.

### PTEN

This gene maps to 10q25 and it encodes a lipid phosphatase that acts as a negative regulator of PI3K pathway by hydrolyzing 3,4,5-PIP3 to 4,5-PIP2. PTEN physically interacts with p53 in the nucleus, leading to p53 stabilization and increased transcriptional activity. PTEN is mutated or deleted in 14% of invasive bladder cancers with 40% LOH at 10q.

### GENOMIC LEVEL ALTERATIONS

Chromosomal aberrations can be primary, related to the cause of a tumor, or secondary, involved in progression. Deletions and gains/amplifications contribute to altered expression of tumor suppressor genes and oncogenes, respectively. Higher rates of genomic alterations are present in pT1 than in papillary pTa tumors. The most consistent alterations in advanced-stage UCC are gains of 1q, 8q and 20q and losses of 8p, chromosome 11 and 9. Array CGH analysis has been applied to the study of UCC. A summary of the most common alterations reported, classified on the basis of their chromosomal location, follows.

#### Alterations common to both pathways (chromosome 9)

The q arm of chromosome 9 is lost both in low and high grade tumors, suggesting it is a primary event in the genesis of bladder cancer. Losses at 9q cover 5 major deleted regions (9q22, 9q32-33, and 9q34) and one or several tumor suppressor genes may be located in them. Candidate genes therein include Netrin, TSCI, PTCH and DBCC1. Allelic loss at 9q has been reported as an early occurrence in the development of bladder cancer but it has also been associated with invasive disease and with disease recurrence in superficial bladder tumors. Deletions of 9p are also common in bladder tumors and affect mainly 9p21, where the INK4A/ARF locus maps.

#### Other alterations -- invasive tumors

Alterations in chromosome 8 often involve loss of the p arm, gain of the q arm and amplification of a small region at 8p12. LOH in 8p is associated with a more aggressive tumor phenotype indicating the possible presence of a tumor suppressor gene. The minimal region of 8p21-22 covers several candidate genes: TRL1, DRB2 and LZTS1. A commonly gained region in 8q contains c-myc. 8p12 amplicons contain FGFRI.

5p amplification is one of the few alterations occurring more frequently in muscle invasive tumors than in early invasive cancers. The most common site of amplification (5p12) contains TRIO; 5p13-12 has also been defined as a critical region of allele losses associated with tumor progression and a marker of adverse prognosis independent of stage and grade. N.M. Luis et al.: Molecular biology of bladder cancer
Methylation

As in other tumors, promoter methylation constitutes a common mechanism of silencing of tumor suppressor genes in UCC. The frequencies of methylation of the best studied genes are: cadherin-1 (36%), RAS-associated domain family (RASSF1A) (35%), CDH13 (29%), secreted Frizzled-related protein 1 (sFRP1) (29%), FHIT (16%), retinoic acid receptor β (15%), p16/INK4A (7%), and death-associated kinase (4%) See. Hypermethylation of APC, p14ARF and RASSF1A has also been described in exfoliated cells in the urine of patients with UCC. A recent study showed that methylation of promoter regions of p16, p14, E-cadherin, RARβ2, RASSF1a and GSTP1 occurs in both normal and CIS samples from patients with UCC and increases with progression. sFRP gene silencing by methylation has been shown to be associated with invasive bladder cancer and to overall survival

Expression Analyses with Microarrays

The first study analyzing gene expression in UCC arrays showed different gene expression profiles in superficial and invasive tumors. The same group subsequently identified expression profiles distinguishing stages, as well as a similar expression profile between CIS and invasive tumors. Data from microarrays with 10568 cDNAs allowed to identify 25 genes able to classify tumors as superficial or invasive with 90.5% accuracy. This classifier had an 82.5% accuracy when used on the data set from Dyskot et al. The gene-classifiers reported by Blavari and Dyskot had no genes in common. Other studies have also reported minimal overlap for the genes identified for clinically similar tumors. Sánchez-Carbayo et al. separated superficial from invasive tumors with 82.2% accuracy and stratified tumors on the basis of clinical outcome with 82% (all tumors) or 90% accuracy (when considering only invasive tumors).

Apoptosis

In the bladder, as in other tissues, failure of the regulatory genes involved in apoptosis may result in survival of cells with genomic abnormalities, tumorogenesis and resistance to anticancer agents. Low levels of FAS and FASL have been associated with higher grade, stage and a poor prognosis. Overexpression of the antiapoptotic protein BCL-2, involved in mitochondrial permeabilization, is associated with p53 overexpression and with poor outcome. Survivin, an inhibitor of caspase-3 and caspase-7, is detectable in urine and has been proposed as a biomarker for the detection of bladder cancer. FHIT protein has been shown absent or reduced in 61% of UCC and its expression correlated with pathological and clinical status.

Invasion and Metastasis

Cadherins are the main mediators of cell-cell adhesion in epithelia. Loss of E-cadherin has been described at higher frequency in high-grade, invasive, UCC than in low-grade papillary tumors. Hypermethylation of CpG dinucleotides in the promoter of CDH1 (encoding E-cadherin) occurs frequently in UCC. The status of E-cadherin has been proposed as an independent prognostic indicator for disease progression.

Concluding Remarks

The information accumulated in the last few years on the molecular changes associated with papillary and invasive bladder tumors allows a more accurate molecular classification of UCC. This information may help not only in the prediction of patient outcome but in the selection of treatment, as well. Bladder cancer is one of the solid tumors in which molecular studies may soon become part of standard clinical practice.

Acknowledgements

Work in the authors’ laboratory was funded, in part, by grants C03/010 and G03/174 from Instituto de Salud Carlos III, Ministerio de Sanidad, Madrid. N.M.L. is recipient of a Ph.D. grant from Fundação de Ciência e Tecnologia, Portugal, and E.L. was supported by a Predoctoral Fellowship from the Ramón Areces Foundation.
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