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REVIEW ARTICLE

Molecular markers in bladder cancer: Novel research frontiers

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Abstract

Bladder cancer (BC) is a heterogeneous disease encompassing distinct biologic features that lead to extremely different clinical behaviors. In the last 20 years, great efforts have been made to predict disease outcome and response to treatment by developing risk assessment calculators based on multiple standard clinical–pathological factors, as well as by testing several molecular markers. Unfortunately, risk assessment calculators alone fail to accurately assess a single patient's prognosis and response to different treatment options. Several molecular markers easily assessable by routine immunohistochemical techniques hold promise for becoming widely available and cost-effective tools for a more reliable risk assessment, but none have yet entered routine clinical practice. Current research is therefore moving towards (i) identifying novel molecular markers; (ii) testing old and new markers in homogeneous patients' populations receiving homogeneous treatments; (iii) generating a multimarker panel that could be easily, and thus routinely, used in clinical practice; (iv) developing novel risk assessment tools, possibly combining standard clinical–pathological factors with molecular markers. This review analyses the emerging body of literature concerning novel biomarkers, ranging from genetic changes to altered expression of a huge variety of molecules, potentially involved in BC outcome and response to treatment. Findings suggest that some of these indicators, such as serum circulating tumor cells and tissue mitochondrial DNA, seem to be easily assessable and provide reliable information. Other markers, such as the phosphoinositide-3-kinase (PI3K)/AKT (serine–threonine kinase)/mTOR (mammalian target of rapamycin) pathway and epigenetic changes in DNA methylation seem to not only have prognostic/predictive value but also, most importantly, represent valuable therapeutic targets. Finally, there is increasing evidence that the development of novel risk assessment tools combining standard clinical–pathological factors with molecular markers represents a major quest in managing this poorly predictable disease.

Keywords

Molecular biology, pathology, statistical analysis, urinary malignancy

History

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Introduction

Bladder cancer (BC) is the most common malignancy of the urinary tract, representing the seventh most common cancer in men and the 17th in women and displaying a 4.5:1 male-to-female ratio[1]. In the European Union, its age-standardized incidence and mortality rate are almost 3-fold of those recorded worldwide and have not significantly changed over the last 30 years[1].

One potential explanation for such inability to reduce both the incidence and the mortality rate of this disease is the heterogeneity of its biologic features leading to extremely different clinical behaviors. In clinical practice, on one hand, as much as 75% of patients present non-muscle-invasive bladder cancer (NMIBC), which has a high recurrence rate but a low yet unpredictable progression rate; on the other hand, patients with muscle-invasive bladder cancer (MIBC) are at high risk for progression and cancer-specific mortality but, again, disease behavior is unpredictable.

In the last two decades, great efforts have been made to identify prognostic and predictive factors for bladder cancer (BC); the former are patient characteristics that can be used to estimate the chance of recovery from a disease or the chance of the disease recurring independently after treatment, whereas the latter are patient characteristics that can be used to estimate the chance of responding to a specific treatment or the chance of developing a condition/disease. Overall, NMIBC has certainly received more attention than MIBC[2].

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As for NMIBC, efforts to identify prognostic and predictive factors resulted into the creation of two risk calculators for disease recurrence and progression, namely the European Organization for Research and Treatment of Cancer (EORTC) and the Club Urológico Español de Tratamiento Oncológico (Spanish Urological Oncology Group, CUETO) scoring systems[6,7]. Both are based on clinical and pathological factors; the EORTC includes number of tumors, tumor size, prior recurrence rate, T category, presence of concurrent carcinoma *in situ* (CIS), and tumor grade, whereas the CUETO does not include tumor size but includes sex and age. The reliability of both calculators has recently been questioned. Xylinas et al.[8] demonstrated that both calculators tend to overestimate the risk of disease recurrence and progression in high-risk patients but their study was strongly biased by its retrospective nature, the multi-institutional design, and the intrinsic heterogeneity of the analyzed data. Covnersely, Palou et al.[9] tested several clinical and pathological factors in predicting recurrence and progression in a homogeneous population of T1G3 BCs treated with Bacille Calmette-Guérin (BCG) (induction, no maintenance) after a complete transurethral resection (TUR) (but without a second TUR); surprisingly, factors like tumor size, number of tumors, and even concomitant CIS were found to have no predictive value, whereas female gender and CIS in the prostatic urethra were found to be the only significant predictive factors.

In the last decade, great efforts have also been made to test the prognostic and predictive role of molecular markers in NMIBC. Again, most studies were strongly biased by the heterogeneity of included tumors, in terms of stage and grade, and included patients, in terms of management; the few studies testing the role of molecular markers in populations homogeneous for tumor characteristics and treatment schedule, however, showed that some markers are reliable and could therefore be considered ready for clinical use[2–5].

Along with the identification of novel molecular markers, current research is exploring the possibility of further improving prediction of disease recurrence and progression by combining molecular with clinical–pathological markers[10,11]. Accordingly, current EAU Guidelines on NMIBC state that ''more work is required to determine the role of molecular markers in improving the predictive accuracy of the currently existing risk tables''.

This review aims to analyze the emerging body of literature concerning novel molecular markers that hold promise to prognosticate disease outcome, predict response to currently available treatments and, possibly, indicate novel pathways for a targeted therapy. The review also aims to present the rationale and the current state of emerging predictive tools, including risk calculators, nomograms, and artificial neural networks, which combine several factors to further improve the single patient risk assessment.

Methods

A PubMed/Medline search was carried out to identify original articles, review articles, and editorials dealing with novel markers for prognosticating BC outcome and predicting its response to available treatments, as well as those dealing with predictive tools in BC. Search keywords included urothelial carcinoma, bladder cancer, transitional cell, biomarker, marker, cystectomy, recurrence, progression, survival, prediction, prognosis, nomogram, artificial intelligence, epigenetics, and genomics. The search was limited to papers published in English, independently of the time of their publication; those with the highest level of evidence were selected and reviewed.

Blood-based markers

Although blood samples offer several advantages over tissue samples (i.e., higher sample homogeneity, time-independent access, and minimally invasive nature), some features, such as dilution effects, the complexity of the serum milieu and proteome, and unreliable tissue-specificity[12,13], make blood-based biomarkers more suitable for BC prognosis and follow-up than for diagnostic purposes. The lack of large multi-institutional prospective controlled trials to date, accounts for the fact that such tests are not currently available in clinical practice.

The characteristics and functions of the blood biomarkers investigated in BC are described below; their prognostic/predictive value is summarized in Table 1.

Insulin-like growth factor (IGF) and its high-affinity ligand IGF binding protein-3 (IGFBP-3) are involved in cell growth and regulation of apoptosis; the latter seems to exert pro-apoptotic effects also directly, irrespective of its binding function and interactions with several molecules such as P53, tumor growth factor beta-1 (TGF beta1), and tumor necrosis factor alpha (TNF alpha)[14]. Serum levels of IGFBP-3, as well as the IGFBP-2/IGFBP-3 ratio assayed on tissue specimens by reverse transcriptase polymerase chain reaction (RT-PCR) showed significant association with clinical and outcome parameters of cancer progression in BC specimens[15,16].

TGF-beta1 has mainly antiproliferative, immunomodulating, and antiangiogenetic functions; its inactivation, often along with loss of expression of its receptors TGF-beta1-RI and TGF-beta1-RII, has been found in association with advanced BC, although conflicting data exist in the literature[17,18].

The matrix metalloproteinases (MMPs) are a family of zinc-dependent proteases involved in tumor progression at different levels, from angiogenesis to invasion and metastasis[19–22]; the ratio between MMP-2 and its tissue inhibitor TIMP-2 seems to be an independent predictor of BC recurrence[23].

Urokinase-type plasminogen activator (uPA) is a serine protease involved in angiogenesis, coagulation, bone modeling, and activation of metalloproteinases and growth factors; in tumorigenesis, it plays a role in tumor invasion and metastasis. The inactive precursor of uPA is activated by binding to a specific membrane-bound or soluble cell surface receptor (uPAR), as confirmed by *in vitro* experiments with BC cells[24,25].

Interleukin-6 (IL-6) is a cytokine that acts as an immune modulator, interacting with cytotoxic T cells, B cells, and acute phase proteins, via its binding to the ligand-specific non-signaling receptor IL-6R. Available data support the hypothesis that BC cell lines produce more IL-6 than normal urothelial cells[26].

Table 1. Predictive potential of selected blood-based markers.

Marker	Study	Method	Significant association	Independent predictor
IGFBP-3	Shariat et al.[15]	DSL-enzyme-linked immunosorbent assays	LN metastases, BC progression, CSS	–
TGF-beta1	Eder et al.[17]	Enzyme-linked immunosorbent assay	Tumor grade	–
	Shariat et al.[18]	Quantitative sandwich enzyme immunoassay	LN metastases	LVI, LN metastases, DR, DSS
	Castillejo et al.[250]	SNP genotype assays	DSM in MIBC (polymorphism TGFBR1-rs868)	–
MMP	Gohji et al.[23]	One-step sandwich EIA system	DR,DFS (MMP-2/TIMP-2 ratio)	DR (MMP-2/TIMP-2 ratio)
	Guan et al.[251]	Enzyme-linked immunosorbent assay	Tumor stage, tumor grade, LN metastases (MMP-9)	–
	Svatek et al.[252]	Multiplexed, particle-based flow cytometric assay	Time to cancer-related death (MMP-7)	–
	Szarvas et al.[253]	Quantitative real-time PCR	MFS, DSS (MMP-7)	–
	Szarvas et al.[254]	Enzyme-linked immunosorbent assay	–	OS, DSS (MMP-7)
uPA, uPAR	Shariat et al.[25]	Enzyme-linked immunosorbent assay	LN metastases (uPAR)	LN metastases, LVI, DP, CSM (uPA)
IL6, IL6R	Andrews et al.[255]	Enzyme-linked immunosorbent assay	muscle invasion, LVI and LN metastases (IL6 and IL6R); tumor grade (IL6R)	LVI, LN metastases, DR, DSS (IL6 and IL6R)
sE-cadherin	Matsumoto et al.[28]	Enzyme-linked immunosorbent assay	LN metastases	LN metastases, DP
CTC	Gallagher et al.[31]	CellSearch system	Metastases (association between number of CTC and number of metastatic sites)	–
	Gradilone et al.[32]	Survivin mRNA isolation	DFS	DFS
	Rink et al.[33]	CellSearch system	OS, PFS, CSS	–
	Guzzo et al.[30]	CellSearch system	–	–
	Rink et al.[256]	CellSearch system	DR, CSM, OM	CSM, OM
	Gazzaniga et al.[257]	CellSearch system	Shorter time to first recurrence, concomitant CIS, tumor stage	–

LN, lymph node; CSS, cancer-specific survival; DSM, disease-specific mortality; MIBC, muscle-invasive bladder cancer; LVI, lymphovascular invasion; DR, disease recurrence; DSS, disease-specific survival; MFS, metastasis-free survival; OS, overall survival; DP, disease progression; CSM, cancer-specific mortality; OM, overall mortality.

Soluble E-cadherin (sE-cadherin) is the degradation product of the transmembrane glycoprotein, E-cadherin, involved in calcium-dependent intercellular adhesion, generated by Ca^{2+} ion-dependent proteolytic activity[27]. In a cohort of 50 BC patients undergoing cycstectomy, preoperative plasma levels of sE-cadherin were independent predictors of lymph node (LN) metastases and disease progression[28].

Circulating tumor cells (CTCs) are tumor-derived epithelial cells present through the bloodstream, carrying the ability to form detectable metastases at distant sites. The presence of CTCs in whole blood of BC patients in the pre- and perioperative setting has been evaluated as an early marker of widespread disease; thus, CTCs may be useful for fostering proper therapeutic decisions, or for monitoring subsequent disease recurrence. Nevertheless, the results and conclusions gained from these studies have been contradictory and inconclusive so far[29–33].

Early studies used RT-PCR for CTC detection in the peripheral blood. Amplification targets that were chosen to identify epithelial cells included CK20, uroplakin II, sirvivin, EGFR, and MUC7 mRNAs. This assay qualitatively evaluates the presence of CTCs in BC patients[32,34–40]. A novel technique for separating CTCs from blood is immunomagnetic capture[41].

Currently, one system that is being used clinically to some extent is the CellSearch assay (Veridex LLC), which employs antibodies targeted at epithelial cell markers (EpCAM and cytokeratins 8, 18, and 19). Using the CellSearch assay in a BC study, CTCs were detected in 44% of patients with metastatic disease, and the number of detectable cells correlated with the number of metastatic sites[31]. The advantage of this method is that it is reproducible across different laboratories and it can identify CTCs in various cancer types.

A major concern is that all these methods may lack sufficient sensitivity, and especially specificity, for BC tumor cells. Evaluation of CTCs can confirm tumor diagnosis and identify patients with advanced bladder cancer, but should not be used as an initial screening test. Therefore, the role of CTCs as a marker in metastatic urothelial cancer requires further evaluation[42,43].

Tissue- and urine-based markers

Gene expression and genomic analysis

Several authors have recently studied BC using an all-encompassing approach involving the assay of multiple alterations at the genomic and transcriptional levels that may play a role in diagnosis and in predicting recurrence and progression[44–57] (Table 2). Novel high-throughput technologies include comparative genomic hybridization (CGH), single nucleotide polymorphism (SNP)-based, and microarray-based approaches. Gene microarray allows simultaneously assay of hundreds or thousands of DNA sequences in a tumor sample, in order to identify alterations of selected

Table 2. Results from selected genetic profiling studies in BC.

Study	Number of patients	Cohort	Tissue/ urine	Significant association	Independent predictor
Modlich et al.[60]	34	NMIBC and MIBC	T	A subset of 41 genes (stage, aggressiveness)	–
Blaveri et al.[65]	80	NMIBC and MIBC	T	global gene expression pattern (stage, histotype, prognosis)	–
Sanchez-Carbayo et al.[49]	105	NMIBC and MIBC	T	174-gene expression profile (LNM, OS)	–
Dyrskjot et al.[66]	404	NMIBC and MIBC	T	52-gene classifier (stage, progression in NMIBC); 88-gene progression classifier (PFS, CSS)	88-gene progression classifier (progression)
Schultz et al.[258]	44	NMIBC (Ta)	T	Survivin (recurrence-free survival)	–
Als et al.[67]	30	advanced BC	T	55-gene signature (survival time)	Survivin, emmprin (response and survival after cisplatin-containing chemotherapy)
Ito et al.[259]	27	NMIBC	T	25-gene expression profile including Pak1 (recurrence)	Pak1 (recurrence)
Holyoake et al.[260]	75	NMIBC and MIBC	U	CDC2 and HOXA13 (grade, stage)	–
Mitra et al.[55]	58	NMIBC and MIBC	T	JUN, MAP2K6, STAT3, and ICAM1 (recurrence, survival)	–
Gazzaniga et al.[261]	35	high-grade NMIBC	T	Molecular profile of chemosensitivity to BCG, mitomycin c, anthracyclines and gemcitabine (intravesical therapy 6 months after TURB)	–
Birkhahn et al.[44]	48	NMIBC (TaG2/3)	T	CCND3, HRAS (recurrence); HRAS, E2F1, BIRC5/Surv, VEGFR2 (progression)	CCND3 (recurrence); HRAS, VEGFR2, VEGF (progression)
Lindgren et al.[53]	144	NMIBC and MIBC	T	Gene signature (grade, stage)	Gene expression signature (metastases, DSS)
Kim et al.[262]	80	BCG-treated NMIBC (pT1)	T	424-Gene expression profile (RFS), 287-gene expression profile (PFS)	12-Gene signature (recurrence), 12-gene signature (progression)
Kim et al.[263]	62+118	MIBC	T	USP18, DGCR2 (cancer-specific survival)	–
Mengual et al.[51,264]	341	NMIBC and MIBC	U	12+2 Gene expression signature (tumor aggressiveness)	–

genes in both tissue and urine specimens[58,59]. Such a comprehensive view of genetic aberrations may be particularly useful in BC, due to the well-known complexity of its origin, progression over time by the accumulation of single genetic or epigenetic alterations, and the heterogeneity that often occurs, especially in NMIBC[60–62].

Dyrskjot et al. identified gene expression microarray profiles in NMIBCs significantly associated with pathological stage and disease progression[63,64]. Subsequently, several studies have identified gene signatures that are associated with tumor stage[49], subtype classification[65], disease recurrence, and outcome prediction[49,65,66]. Some of them have been further validated on independent tissue collections[67,68].

A number of studies aimed to develop a molecular signature capable of assessing response to cytotoxic chemotherapy in selected BC cases[67,69]. Using the known gene-expression profiling and drug sensitivity data from the NCI-60 panel of tumor cell lines, Theodorescu et al. have recently developed a bioinformatic approach called Coexpression Extrapolation (COXEN)[70,71], which is able to predict response to neoadjuvant chemotherapy in MIBC.

Of particular interest is a study by Lindgren et al.[53], where the application of gene expression analysis, whole genome array-CGH analysis and mutational analysis of selected genes on a large cohort of BCs, with subsequent validation in two independent data sets, led to the identification of two intrinsic molecular signatures (MS1 and MS2). Their results support the capability of molecular classification, in adjunct to histopathology, to correctly stratify tumors by grade, stage, and outcome parameters; moreover, they provide a gene expression signature that independently predicts metastasis and disease-specific survival.

Therefore, high-throughput genomic analysis holds great promise in BC but is currently quite expensive[72], and the results obtained so far need to be validated by additional clinical trials on multiple independent patient cohorts.

Specific DNA changes

Mitochondrial DNA. Mitochondria are essential organelles in all eukaryotic cell systems as the powerhouse to provide ATP for a multitude of cellular processes by the oxidative phosphorylation (OXPHOS) system. Mitochondria have their own genetic system: the mitochondrial DNA (mtDNA) is a small (16.5 kb), double-stranded, closed circular DNA molecule present in a large number of copies per cell. It codes for 13 subunits of the OXPHOS system, as well as for 2 rRNA and 22 tRNAs.

Warburg hypothesized that a decrease in mitochondrial energy metabolism might lead to cancer development[73]. Mitochondrial dysfunctions, particularly alterations of mtDNA (deletions, point mutations, and mtDNA copy number), are among the factors responsible for a decrease

in mitochondrial energy metabolism and are now actively studied since they may suggest new approaches for diagnosis and therapy of tumors. In particular, detection of mtDNA mutations can be used as a tool for early detection of cancer in clinical samples including body fluids and serum, since it offers a distinct advantage over nuclear DNA because of the high copy number of mitochondrial genomes in cells[74–76]. A high incidence of mtDNA mutations has been observed in BC, suggesting that mitochondria could play an important role in such carcinogenesis and indicating mtDNA as a potentially valuable marker for early diagnosis of BC[77–80].

Interestingly, the analysis of mtDNA from blood, tumoral tissues, and adjacent non-tumoral tissues of 26 patients with BC, and DNA from blood of 504 healthy controls from different ethnicities, demonstrated that a particular mtDNA variation, namely the C16069T variation, was associated with BC[81]. As for mtDNA alterations in body fluids, these have been also detected in urine sediments from BC patients[79,82–85]. Moreover, it was observed that circulating mtDNA levels in serum were significantly increased in cancer patients and allowed sensitive (84%) and specific (97%) discrimination from healthy controls. The mtDNA-integrity (defined as the ratio of the long amplified fragment, mtDNA-220, to the short mtDNA fragment, mtDNA-79) was increased in BC patients compared with control subjects and was correlated with tumor grade[86].

The expression of sense mitochondrial long non-coding RNA (SncmtRNA) and the antisense strand (ASncmtRNAs) was analyzed in exfoliated bladder tumor cells from low- and high-grade BC. It was observed that the cells maintained the expression pattern observed in BC biopsies. In contrast, exfoliated cells recovered from healthy donors revealed no expression of these mitochondrial transcripts. This assay deserves further investigation as a non-invasive diagnostic tool for BC[87]. Taken together, these findings indicate that mtDNA alterations represent an interesting, novel marker, which deserves extensive evaluation in BC.

Another interesting target for research is represented by the mitochondrial GTPase mitofusin-2 gene (*Mfn2*), a novel gene characterized as a cell proliferation inhibitor[88,89]. The *Mfn2* gene is also a mitochondrial protein and is the main regulator of mitochondrial fusion at the level of the outer mitochondrial membrane[90]. It has been reported that this protein showed significantly lower expression in urinary bladder carcinoma (UBCC) tissues compared with nearby non-tumorous tissues. *Mfn2* overexpression in UBCC cells significantly inhibited cell proliferation, by arresting the transition of the cell cycle from the G1 to S phase, and induced apoptosis by upregulating active caspase-3 and increasing cleavage of poly (ADP-ribose) polymerase PARP. These findings indicate that the *Mfn2* gene is a potential UBCC tumor suppressor gene and could promote apoptosis and inhibit the proliferation of UBCC cells. The *Mfn2* gene, therefore, may become a prognostic marker and an important therapeutic target for treating UBCC[91].

Telomerase. Telomerase is a ribonucleoprotein DNA polymerase that repairs the ends of chromosomes (telomeres) by adding tandem repeat sequences (TTAGGG), in order to avoid progressive shortening and ultimately prevent cell senescence and inability to divide. Increased expression of telomerase has been demonstrated as a growth advantage of cancer cells in a variety of organs, including the urinary bladder[92].

Telomerase activity is usually assayed by the PCR-based assay called telomeric repeat amplification protocol (TRAP). Measurement of telomerase activity by the TRAP assay has been proven to be a diagnostic marker for BC in tissue and urine samples[93–104]. This method seems to be limited in diagnosing BC in urine, since reported sensitivities have been widely variable and inactivation of the telomerase enzyme in urine has been shown to compromise the diagnostic yield[105–107].

Detection of the mRNA of the catalytic subunit of telomerase by RT-PCR, called human telomerase reverse transcriptase (hTERT), appears to be a useful alternative. The *hTERT* gene seems to be the rate-limiting determinant of telomerase reactivation, and its expression was reported to correlate with telomerase activity. As a well-known cancer biomarker, hTERT has been proven to be a reliable diagnostic marker for BC as well, in both tissue and urine samples[108,109].

The prognostic role of hTERT has been examined by several authors in the last few years. Brems-Eskildsen et al.[110] analyzed urine samples from 117 BC patients, detecting a significant association of hTERT with tumor recurrence ($p = 0.0001$); this finding has been confirmed in a most recent study by Mucciardi et al[111]. A previous study, however, failed to demonstrate a correlation between telomerase activity and different clinical–pathological features, such as cancer stage, grade, and multifocality, or outcome parameters, such as cancer recurrence[94].

Recently, the recognition of specific somatic mutations at the promoter region of hTERT has shed more light on the biological role of this gene in BC[112,113]. Such mutations, identified in both tissue and urine specimens, have been reported to occur in both papillary and flat lesions, often as early events in tumorigenesis, and to influence patient survival and disease recurrence[114,115]. Allory et al.[116], however, found no correlation between hTERT expression and disease outcome in terms of progression-free survival, disease-specific survival and overall survival, although the authors themselves acknowledged that their study was limited by its retrospective nature.

PI3K/AKT/mTOR pathway molecules. The phosphoinositide-3-kinase (PI3K)/AKT (serine–threonine kinase)/mammalian target of rapamycin (mTOR) pathway is an important cell-signaling pathway regulating growth and survival; thus, this pathway is involved in the development and clinical behavior of several solid malignancies. In urothelial carcinogenesis, it is considered the most important pathway promoting cell growth, along with RAS/MAPK[117–120].

Further evidence is provided by the fact that somatic alterations in all three genes, as well as in PTEN, a tumor suppressor that codes for a lipid phosphatise, which acts as a negative regulator of the pathway (commonly deleted in MIBC)[121–123], have been found in BC[121,122,124] and could influence tumor behavior and disease outcome. Specific SNPs from this pathway (namely AKT2:rs3730050, PIK3R1: rs10515074, and RAPTOR: rs9906827) were reported as significantly associated with survival in a large cohort of MIBC, both as single alterations or in a combined manner[125].

According to Sun et al.[126], the altered expression of PI3K pathway-related proteins in BC was related to clinicopathological and outcome parameters; such findings have subsequently been confirmed by other authors[127]. Moreover, p-Akt and p-mTOR were found to be independent predictors of outcome in the NMIBC and MIBC groups, respectively[126]. On the other hand, a study using multiple-mutation assay failed to reveal a significant association between mutations in the *PIK3CA* gene and outcome parameters, such as recurrence-free, progression-free and disease-specific survival[119].

Taken together, available data suggest the opportunity to test the expression of the PI3K/AKT/mTOR pathway molecules in BC, not only as markers of disease outcome but also as potential therapeutic targets, given the fact that mTOR inhibitors (rapamycin and analogues) are already approved for clinical use in kidney cancer and other malignancies[128].

Epigenetic markers

Epigenetic means changes in the function/expression of a gene without any structural change in its DNA sequence. The most common epigenetic change investigated in BC is DNA methylation, i.e., the addition of a methyl group to the cytosine-5 position of the cytosine–guanine dinucleotide (CpG); this leads to the formation of the altered base 5-methylcytosine, usually in the promoter region[129]. DNA methylation is a physiological mechanism involved in maintaining homeostasis, as it results in stability and transcription of genes. Conversely, abnormal DNA methylation of CpG islands may lead to tumor-suppressor gene silencing, and there is evidence that this often occurs as an early event in several types of solid tumors[130–132].

As for BC, promoter hypermethylation of CpG islands has been significantly associated with tumor development, advanced stage, higher tumor progression rate, and increased mortality rate[133–140], suggesting that tissue-specific methylation status can have relevant diagnostic and prognostic implications[57,133–137,141–155].

Hypermethylated tumor-suppressor genes may effectively be detected in both liquid and tissue specimens by means of different specific techniques, such as methylation-specific polymerase chain reaction (PCR), methylation sequencing, methylation-sensitive endonuclease digestion followed by electrophoretic separation, and methylation-specific multiplex ligation-dependent probe amplification assay (MS-MLPA)[150]. The last method is a novel one, carrying the advantages of requiring only a small quantity of DNA, rapidly determining the methylation status of numerous genes in the same experiment, and working well in formalin-fixed paraffin-embedded samples[156]. The employment of different techniques, each with unique features and accuracy, may, however, represent a structural bias in the evaluation and comparison of their (apparently discordant) results.

Since epigenetic silencing by DNA hypermethylation often involves changes in the interphase chromatin architecture, it is potentially reversible (unlike point mutations), and, therefore, represents a suitable target for cancer treatment. DNA methylase inhibitors, such as 5-aza-2′-deoxycytidine, or histone deacetylase inhibitors, are able to restore the activity of tumor-suppressor genes, and thus modulate cell proliferation, differentiation, apoptosis, and other key homeostatic mechanisms, as well as prevent cells from acquiring further DNA hypermethylation[134–137,141–145,157–161]. The possibility of these drugs being used either alone or in combination with the standard treatment modalities for BC, such as surgery, intravesical immunotherapy, intravesical or systemic chemotherapy, or radiation therapy, deserves further attention.

Most importantly, the hypermethylation of particular genes seems to effectively predict the outcome of BC (see Table 2). Agundez et al. reported the methylation status of 25 tumor suppressor genes to be a reliable predictor of response to BCG therapy in NMIBC[162]. Using multivariate analysis, Yates et al.[137] demonstrated the overall degree of methylation, detected by quantitative methylation-specific PCR (MSP) on a panel including 17 gene promoter regions, to be more significantly associated with higher progression and poorer survival than the tumor stage in BC. Yates' study focused on five loci, namely RASSF1A, E-cadherin, TNFSR25, EDNRB, and APC, as predictors of tumor progression. A previous study by Catto et al.[134] had already highlighted the association of hypermethylation at the RASSF1A, along with DAPK gene promoters, with disease progression, independent of tumor stage and grade, on a series of 280 urothelial (bladder and upper urinary tract) tumors. Ha et al. have recently reported that aberrant methylation of RASSF1A could play a relevant role in predicting recurrence in low grade NMIBC[163].

Other studies have focused on RUNX3, which is a tumor-suppressor gene with peculiar features, in that it is inactivated primarily by epigenetic silencing. Kim et al. first demonstrated that RUNX3 hypermethylation led to a higher risk of developing BC[135] and was positively associated with tumor stage, recurrence, and progression. This suggests that RUNX3 is a tumor suppressor, which not only inhibits cancer initiation but also suppresses the aggressiveness of primary BC[135]. Subsequently, they demonstrated by multivariate analysis that RUNX3 hypermethylation was the only strong predictor of BC progression in a cohort of BC patients with long-term follow-up[143].

Other epigenetic markers that have been tested for their prognostic/predictive value are (i) the activin membrane-bound inhibitor (BAMBI) gene, which is epigenetically silenced in high grade BC and is correlated to high aggressiveness and invasiveness[164]; (ii) the *hDAB2IP*, *SYK*, and *CAGE-1* genes, which are associated with tumor progression[165]; (iii) the actin-binding protein myopodin, which has been found to correlate with an increased risk of recurrence and death in a large series of mostly BCG-treated pT1G3 NMIBC[166].

Interestingly, a most recent study reported a positive correlation between gene methylation and lack of recurrence, highlighting that putative tumor suppressor genes do not always act as tumor suppressors but may actually have different biological functions[156]. Specifically, the combined analysis of three genes (*HIC1*, *GSTP1*, and *RASSF1*) showed 72% accuracy in predicting tumor recurrence[156]. Therefore, care is needed in epigenetic research; it is highly debatable that methylation could be the only basis of NMIBC recurrence[156]. Conversely, it is likely that not only different

markers but also potentially the same marker with different alterations may be involved in the complex mechanism of NMIBC recurrence[57].

To sum up, as for many other putative markers, it is difficult to draw any definite conclusion about the prognostic and predictive role of epigenetic changes in both NMIBC and MIBC due to the fact that the reported studies included tumors of different stages and grades that received different treatments.

MicroRNA markers

Micro RNAs (miRNAs) are a class of small (18–24 nucleotide) non-coding RNA molecules that are endogenous inhibitors of gene function, acting by modulating mRNAs at the posttranscriptional level. miRNAs have been proven to be involved in normal cellular processes, as well as in tumorigenesis. Accordingly, miRNA-based therapies are being investigated in cancer patients.

MiRNA expression profiles may be analyzed by miRNA microarray (for genomewide miRNA expression profiling) or by quantitative PCR/RT-PCR-based techniques (in a targeted manner) in cancer specimens[167–169]. However, several studies have detected miRNAs in urine and serum samples, highlighting two further advantages of this type of marker, i.e., low invasiveness and resistance to nuclease degradation, thus making miRNAs suitable also for diagnostic purposes[170–178].

A growing body of evidence suggests that miRNAs contribute to BC development, progression, and metastasis. Alterations in miRNA levels have been reported to occur often in BC carcinogenesis, representing an early event, and to be associated with tumor grade (up-regulation of miR-21 in high-grade BC versus down-regulation of miR-99a/100 in low-grade tumors)[179]. miRNAs also have been associated with tumor stage (NMIBC versus MIBC)[175,180,181], as well as with tumor aggressiveness, poor outcome, and increased risk of disease[170,180,182,183].

A recent study by Yoshino et al. identified BC-specific miRNA signature sets[184], including both down-regulated (such as miR-145, miR-143, and miR125b) and up-regulated miRNAs (such as miR-183, miR-96, miR17-5p, and miR-20a), acting as tumor suppressors and oncogenes, respectively. miRNAs are involved in the regulation of multiple functions including apoptosis, cell-cycle progression, and epithelial–mesenchymal transition by controlling crucial molecules, such as P53 and FGFR3, at the gene expression level. The predictive value of miRNAs has been tested only in MIBC whereby specific miRNA expression profiles have been shown to be associated with both cisplatin response and survival[185].

Proteomics

The study of protein structure and function in fluids (serum and voided urine) or tissues from BC patients, also called proteome profiling, has been extensively investigated in the last few years[59]. Besides its use as a putative diagnostic alternative to urine cytology[186,187], proteomics has been exploited with the goal of identifying factors that can further stratify tumors or predict their outcome. The search for new urine biomarkers by proteome profiling parallels the development of novel methods, from classical two dimensional gel electrophoresis (2-DE) followed by mass spectrometry[188–191], to novel gel-free approaches, namely shotgun proteomics and capillary electrophoresis coupled to mass spectrometry (or ''peptidomics'')[192], and ultimately to more specific tests (i.e., protein binding assays, or protein microarrays), which are suitable in the phase of biomarker verification along with Western blotting and immunohistochemistry[192].

The profiling of BC samples through new technologies led to the identification of specific proteins/peptides out of a proteome panel. Further validation on tissue specimens showed that some of these proteins/peptides may be able to differentiate, with high accuracy, NMIBC from MIBC[193–195], and to predict disease outcome[190,196,197]. Since the combined application of proteomic and genomic urine sample profiling data has already provided interesting results as a diagnostic tool in BC[198–201], this may represent a promising approach in the discovery of effective predictive markers.

Hormone receptors

Since gender is a well-known prognostic factor in BC[202,203] that has already been incorporated in the Bladder Cancer Research Consortium (BCRC) and International Bladder Cancer Nomogram Consortium (IBCNC) nomograms for predicting patients' outcome after RC[204], hormone receptors (HRs) have been studied as putative molecular predictors, as well as targets of tailored therapies. As a result, there has been increasing evidence that nuclear steroid HRs and ligand-dependent transcription factors, such as androgen receptors (AR), estrogen receptors (ER), and progesterone receptors (PR), are engaged in BC tumorigenesis[205–207], mainly due to their ability to initiate multiple signaling pathways on ligand binding, eventually leading to cell change.

The AR is often expressed in BC as compared with benign urothelium[205–211]. Its role as an outcome predictor in BC has been analyzed in a few studies, leading to conflicting results: while some authors reported a significant association with grade and stage[205,207,210–213], others failed to demonstrate a relation with clinical–pathological features and outcome parameters[214–216]. Although Miyamoto et al. demonstrated in several studies that AR signaling is an inducer of BC tumorigenesis, and that androgens are able to promote the growth of AR-positive BC cells, the exact role of AR expression in BC progression, as well as its potential as a prognostic-therapeutic marker remains controversial[205,206,215,216].

The ER exists in two subtypes with varying expression and functional profiles, alpha (or ESR1) and beta (or ESR2). ER-alpha has been rarely detected in BC, and its expression seems to be associated with stage[217]. Expression of ER-beta has been associated with stage and grade in BC[218,219], but different studies failed to show an impact of ER expression on BC outcomes[212,217,219]. In two recent studies, ER-beta expression was associated with better recurrence-free survival in a series of 42 NMIBCs[220], and with recurrence, progression, and overall disease-specific mortality in BCs of different grades[205].

Although its expression has been detected in benign urothelium[221,222], the PR has not been demonstrated in BC specimens so far[216,217].

Multimarkers statistical models

The high number of molecular studies on BC in the last several years has led to a huge amount of results regarding the putative predictive role of single molecules and/or specific genome/proteome profiles. It has become clear that a multimarker panel is likely to provide a more accurate risk stratification and outcome prediction than any single marker[223–228].

The most recent molecular studies tend to include multivariate analyses of clinical–pathological features and novel markers, often along with area-under-the-curve analyses, to better define the statistical value of adding a putative marker(s) to the standard clinical–pathological indicators. Moreover, attempts have been made to include novel markers into advanced statistical models to determine whether the addition of a putative marker to the standard clinical–pathological markers provides further advantages in terms of risk stratification and outcome prediction.

Nomograms are graphical representations of mathematical formulae or algorithms incorporating several prognostic factors as continuous variables to predict a particular end point[229]. Following validation in independent patient cohorts, nomograms are expected provide superior outcome prediction for the individual patient than risk group classifications[230,231], as they more reliably account for the multistep nature of tumor recurrence and progression[232]. For instance, the pre-cystectomy nomogram developed by Karakiewicz et al. for advanced BC using patient age, stage, grade, and the presence of CIS has been shown to be more accurate than the TUR stage in predicting T and N variables[233].

Several post-cystectomy nomograms have been developed to predict the natural outcome of surgically treated BC and to assist in deciding on the use of adjuvant therapy after RC. The two main multi-institutional models for predicting outcome after RC include the International Bladder Cancer Consortium (IBCC) Nomogram, based on the analysis of traditional clinico-pathological parameters (age, grade, pathological stage, histological type, LN status, and time from diagnosis to surgery)[234], and the BCRC, which includes a wide spectrum of variables (pathological T and N stages, grade, LVI, CIS, and the utilization of neoadjuvant or adjuvant chemotherapy and/or radiation)[235,236]. Both were significantly better predictors than TNM staging for 5-year recurrence and disease recurrence, cancer-specific mortality, and all-cause mortality nomograms[237]. Such nomograms have been cited in the latest EAU guidelines[238], but their use has received only a grade B recommendation (level of evidence 3) in clinical practice, due to the lack of external validation.

The predictive accuracy of nomograms has been shown to be due to the incorporation of molecular markers, such as urinary NMP22 for predicting disease recurrence and progression in NMIBC[239], or cell-cycle molecular markers (p53, pRB, p21, p27, and cyclin E1) for recurrence and cancer-specific mortality in BC patients undergoing cystectomy[224,225].

Artificial intelligence (AI) methods such as artificial neural networks (ANN), an algorithm-based pattern[240], and neurofuzzy modeling (NFM) have been applied to BC outcome parameters in a few studies. Comparing AI methods with other complex models, the first AI methods outperformed conventional statistics and nomograms in predicting 5-year survival after RC in MIBC[241], and relapse and time to relapse in a cohort of NMIBC and MIBC[242].

Finally, the CO-eXpression ExtrapolatioN (COXEN) model is an algorithm-based system developed on cell lines from different tumors (bladder, breast, and ovary) in order to predict response to chemotherapy[71,243], and for *in silico* drug discovery[71,244].

Potential limitations of implementing bladder cancer biomarkers

As shown above, novel biomolecular techniques have allowed continuous discovery of novel putative prognostic and predictive markers for BC. The positive aspect of this continuous search is the possibility of finding the ''ideal'' marker, which theoretically, should be expressed uniquely by the malignant tissue and should not be biased by tumor heterogeneity; thus, the ideal marker should be able to distinguish between aggressive and non-aggressive tumors. Finally, markers should have a high prognostic/predictive accuracy while being cost-effective. The negative aspect of the continuous search for novel biomarkers could be dedicating little attention to those that have already shown to be effective in well-conducted pilot studies. In other words, there could be a risk of dissipating research energies in the search for novel markers before having fully validated those that could soon become ready for use in routine clinical practice.

Conclusions

BC is a complex and heterogeneous disease encompassing several factors, ranging from genetic changes to altered expression of a huge variety of molecules, including chromosomal markers, genetic polymorphisms, and genetic and epigenetic alterations, which may all be potentially involved in tumorigenesis, disease progression, and determining patient outcome.

Recent data have clearly shown that current risk scores based on traditional clinical and pathological variables provide important but limited prognostic information, in terms of disease outcome, and predictive information, regarding response to available treatments. On the other hand, a better understanding of the molecular mechanisms involved in carcinogenesis and cancer progression has led to the identification of a large number of putative molecular markers of BC with reliable prognostic/predictive value[2,245–249]. Such markers include DNA, RNA, and proteins and may be assayed in different biological samples, such as serum, urine, and tissue. It has also become clear that the most efficient prognostic signatures are likely made up of combinations of independent, complementary biomarkers, rather than based on a single indicator.

In spite of such efforts and the resulting encouraging data, differences in study designs and patient cohorts, inconsistent immunohistochemical staining criteria, different cut-off points in result reporting, and variable outcome parameters, have accounted for the difficulties in comparing individual studies and the lack of consensus regarding the role of the

identified markers in clinical practice. Moreover, our knowledge of BC initiation and progression remains far from complete.

Novel research frontiers should therefore move towards three directions. The first remains the identification of further molecules that could reliably prognosticate tumor behavior and/or predict tumor response to treatments, with the possibility of developing concise pathway-based multimarker panels. In this respect, it is essential for the markers not only to have high sensitivity and specificity but also to be easily assessable in a fast and cost-effective manner with currently available standard technologies. The second is the optimization of available markers by means of their incorporation, together with the standard clinical–pathological indicators, into sophisticated statistical tools such as AI technologies and nomograms. These tools have already provided better individualized risk estimates, but still need to be systematically evaluated in large, possibly prospective, clinical trials. The third, more fascinating direction, is the identification of reversible alterations so that the impaired cell function and eventually the cancer phenotype could be restored by ''smart'' drugs, such as DNA methylase or histone deacetylase inhibitors, used alone or in combination with conventional drugs.

This review aimed to ''open a window'' to a number of novel molecules that, to date, hold promise but are not established markers for BC. Researchers are called to the difficult task of continuing the search for novel biomarkers, as well as identifying, among the plethora of available ''putative'' markers, those that really deserve an extensive validation process, keeping in mind that the final goal is to provide information that could be translated into a patient-tailored and tumor-targetted approach.

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