Bladder Cancer Tissue-Based Biomarkers

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Abstract

This review aims to provide a practical update regarding the current role of tissue-based biomarkers in bladder cancer. Their prognostic and predictive role both in non-muscle-invasive (NMIBC) and in muscle-invasive disease (MIBC) has been reviewed with particular focus to their use in clinical practice.

In summary, the literature on the prediction of disease recurrence in NMIBC is inconclusive, and there is little information on prediction of response to intravesical bacillus Calmette-Guérin (BCG).

Concerning disease progression, external prospective validation studies suggest that FGFR3 mutation status and gene signatures may improve models that are based only on clinicopathologic information.

In MIBC, tissue-based biomarkers are increasingly important, since they may predict the response to systemic chemotherapy and immunotherapy. In particular, the advent of molecular characterization promises to revolutionize the paradigm of decision-making in the treatment of MIBC. Molecular subtyping has been shown to improve the prediction of pathological stage at RC and to predict the response to systemic chemotherapy and immunotherapy. However, external and prospective validations are warranted to confirm these preliminary findings.

Several different tissue-based biomarkers such as PD-1/PD-L1 expression, tumor mutational burden, and the analysis of tumor microenvironment, may in future play a role in selecting patients for systemic immunotherapy. However, to date, no pretreatment recommendations can be definitively made on the basis of any molecular predictors.

In conclusion, despite the potential of tissue-based biomarkers, their use in bladder cancer should be limited to experimental settings.

Introduction

In recent years, there have been significant innovations in the treatment of bladder cancer (BCa). While most treatments are standardized, we are transitioning from the era of "one size fits all" into the era of "precision medicine," in which treatments are personalized and tailored according to the particular characteristics of each patient and tumor. Biomarkers play an undeniable role in this setting, allowing patient risk stratification, predicting response to treatments, and paving the way for targeted therapies. In this non-systematic review, the technical aspects of tissue-based biomarkers, as well as their current role in terms of clinical utility, both in non-muscle invasive (NMIBC) and muscle-invasive bladder cancer (MIBC), are reviewed.

Key Words

Bladder cancer, tissue-based biomarkers, prognosis, prediction, immunotherapy

Competing Interests

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Abbreviations

AUA	American Urological Association
BCa	bladder cancer
CSM	cancer-specific mortality
EAU	European Association of Urology
MIBC	muscle invasive disease
NAC	neoadjuvant chemotherapy
NMIBC	non-muscle invasive bladder cancer
RC	radical cystectomy

Technical Aspects of Tissue-Based Biomarkers in Bladder Cancer

Several technical aspects should be considered when working with tissue specimens. First, there are important differences between using fresh, frozen, or formalin-fixed material. The use of fresh or fresh-frozen tissue, for example, enriches the quality of RNA-derived material as compared with formalin-fixed tissue[1]. Conversely, DNA is usually more stable, and unless long nucleic acids are required, DNA of sufficient quality for analysis can be obtained for the majority of the PCR-based methods in fresh, fresh-frozen, or formalin-fixed material.

Second, it is critical to consider that BCa is extremely heterogeneous. This is particularly evident in MIBC, in which tissue heterogeneity might be extremely high, with differential ratios between tumor and stromal cells among paraffin blocks, leading to very different results if microdissection is not performed. This may lead to significant differences when staining sections from different blocks. However, even when microdissection is performed, intratumoral heterogeneity may currently play a role in limiting the use of tissue-based biomarkers in BCa.

Third, sample handling is very important to preserve the quality of the specimen. In frozen material, it is important that freezing is performed within 30 minutes of the specimen's removal. When working with paraffin-embedded tumors it is important that specimens are maintained no longer than 24 hours in formalin for fixation and that blocks are well orientated to be cut within that time[2,3].

Finally, the use of controls is mandatory. A comparison with normal urothelium should always be performed in any experiment at the DNA, RNA, or protein level[4]. This is not always possible for BCa because of the field effect of carcinogenesis so adjacent "normal" may be best alternative control.

Tissue-Based Biomarkers in Non-Muscle Invasive Bladder Cancer

In general, prognostic information for patients with NMIBC is highly desirable, because guidance for further treatment and follow-up is urgently needed. So far, this information relies exclusively on clinico-pathological parameters[5–8]. Since there is a broad range of recurrence and progression rates even when applying European Association of Urology (EAU) or American Urology Association (AUA) risk stratification, there is need to refine prediction of rates to help inform treatment and surveillance decisions. Key prognostic biomarkers for disease recurrence and progression in NMIBC are listed in Table 1 and are briefly described below.

Prognostic markers for disease recurrence

The p53 tumor suppressor gene is probably the first molecular alteration that has been extensively studied, but there have been conflicting results regarding association with prognosis[9–14]. Using p53 as a single marker has issues due to multiple cell cycle regulators that may have overlapping roles. Considering the biological diversity of NMIBC along with its intratumoral heterogeneity, the research focus in tissue marker research has shifted from the investigation of single alterations to consideration of combined alterations, including gene classifiers for discrimination between recurrent and non-recurrent NMIBC, with promising results[13,15–17]. Nevertheless, a large international retrospective validation study including 404 patients investigating a 26-gene signature found no association with tumor recurrence[18].

FGFR3 mutations have been correlated with the prognosis of patients with NMIBC. A retrospective multicentre study investigated the prognostic potential of FGFR3 status and 3 molecular markers (MIB-1, P53, and P27kip1) showed that the combination of FGFR3 and MIB-1 was able to independently predict disease recurrence[12]. More recently, dysregulation of several miRNAs has been suggested to predict tumor recurrence[19–21]; however, validation and prospective assessment are lacking.

In summary, the role of molecular markers in the prognostication of disease recurrence in NMIBC seems limited, not only for technical reasons but also because clinical parameters (eg, multiplicity, tumor size, incomplete TUR) have a substantial effect on this event and mitigate the impact of biomarkers[12]. Furthermore, most patients with NMIBC, especially those with high-risk disease, undergo treatment, and response to therapy will impact the likelihood of recurrence significantly.

TABLE 1.

Prognostic biomarkers in non-muscle invasive bladder cancer

Molecular pathway	Biomarker(s)	Method	n	Results	Year	Reference
Cell cycle regulation	Rb	IHC	74	No association with progression	1996	96
	P21	IHC	207	No association with progression	2000	97
			244	No association with recurrence	1999	98
	P27	IHC	61	No association with recurrence and progression	2013	99
	Ki-76	IHC	61	No association with recurrence and progression	2013	99
Cell death pathways	p53	IHC	69	Overexpression predicts disease progression	1995	9
		IHC	104	Overexpression predicts disease recurrence	1997	10
		IHC	286	Overexpression alone predicts disease progression, but not in combination with FGFR3 mutation	2003	12
		IHC	83	Overexpression predicts disease recurrence and progression	2007	13
		rtPCR	105	No prognostic value	2007	14
	Bcl-2	IHC	100	No association with recurrence	1998	100
		IHC	93	No association with recurrence	2000	101
Cell growth signaling	FGFR3	rtPCR	286	Mutation associated with higher recurrence-free and progression-free survival	2003	12
	erbB2 (HER2)	IHC	88	Association with recurrence and progression	2015	102
		rtPCR	141	Association with recurrence and progression	2015	103
		rtPCR	34	Association with progression	2017	104
	Survivin	IHC	233	Association with progression	2016	105
			115	Association with recurrence and progression	2015	106
			283	Association with progression and survival	2012	107
Angiogenesis markers	VEGF	IHC	185	No association with recurrence	1999	108
			140	No association with recurrence and progression	2005	109
	HIF-1 α	IHC	140	No association with recurrence and progression	2005	109
Immune markers	PD-L1	rtPCR	296	Association with recurrence, progression, and survival	2018	110

IHC: immunohistochemistry

Prognostic markers for disease progression

Progression of disease is defined as a recurrence with a worsening stage or grade of disease. p53 alteration is one of the first and certainly most frequently studied markers in this context. Most of these studies, including a combined analysis of 23 studies[22], reported a correlation between p53 overexpression and tumor progression. However, as p53 alterations are closely related to tumor grade, stage, and other molecular changes, the independent prognostic value of this parameter remains a matter of controversy[11–13]. Immunohistochemical p53 overexpression has also been tested in combination with other alterations, frequently related to cell cycle regulation. In one prospective study every patient with high-grade NMIBC underwent immunohistochemical staining for 5 biomarkers (p21, p27, p53, KI-67, and cyclin E1) no differences were found in progression or survival based on the number of altered markers[23].

A molecular grading based on the combination of FGFR3 mutation together with MIB-1 expression is significantly associated with disease progression. A large prospective study of 1239 patients from the same group demonstrated that molecular grading based on FGFR3 mutational status and methylation of GATA2 was able to improve the EAU NMIBC risk score in predicting tumor progression[24].

The development of gene classifiers and subtyping using microarrays is another option for combining molecular information[25,26]. In a large prospective Scandinavian-based trial of 1224 patients, Dyrskjøt et al. demonstrated that the results of a 12-gene real-time qualitative PCR assay yielded independent prognostic information on tumor progression[27]. Nevertheless, with a 66% sensitivity and specificity to predict tumor progression as a stand-alone assay, it becomes obvious that, at this stage, information obtained by molecular markers is not sufficient and needs to be integrated with established clinicopathologic variables.

Predictive markers for response to intravesical therapy

Various tissue-based biomarkers have been evaluated for prediction of response to intravesical bacillus Calmette-Guérin (BCG) therapy. To date, the best evidence comes from a validation study based on 2 Nordic multicenter trials comparing treatment with BCG and other intravesical adjuvant therapies[28]. In this report, ezrin, CK20, and Ki-67 have been analyzed in a tissue microarray: unfortunately, none of the variables correlated with disease recurrence, and only tumor multifocality was associated with disease progression.

Several studies demonstrated a clinical utility in combining gene expression signatures with clinicopathologic features[29]. Pietzak et al. demonstrated that patients with NMIBC had a high prevalence of alterations to DNA damage repair genes and that mutations in *ARID1A* are associated with an increased risk of recurrence following BCG therapy[30]. Moreover, total mutational burden has been associated with disease progression in a small retrospective study of 25 patients treated with BCG[31].

Following the advent of immunotherapy with checkpoint inhibitors also in the NMI setting (recently, according to the results of the Keynote-057 trial[32], the use of pembrolizumab in patients with BCG-unresponsive Cis has been approved by the FDA), the role of potential immune markers (ie, PD-1 and PD-L1 mRNA expression) to predict response to BCG has been investigated with promising results. However, it should be underlined that these findings need to be externally validated before they could be considered for clinical practice. Finally, in the context of immunological markers, Pichler et al. studied the association between recurrence-free survival and the count of CD4, GATA3, tumor-associated macrophages, Tregs, and T-bet+ T cells in the malignant tissue samples prior to BCG therapy[33]. They found that CD4+ and GATA3+ T cells were predictors of prolonged recurrence-free survival, while the predictors of shorter recurrence-free survival were TAMs, Tregs, and T-bet+ T cells.

Tissue-Based Biomarkers in Muscle-invasive Bladder Cancer

Prediction of Oncological Outcomes

The standard treatment for patients with MIBC is radical cystectomy (RC) with neoadjuvant chemotherapy (NAC). However, despite the administration of adequate therapy and the recent development of new treatment strategies such as trimodal therapy (TMT) or targeted therapies, MIBC remains an aggressive disease characterized by a generally unfavorable prognosis [34,35]. There are significant challenges in accurately staging the disease and insufficient ability using clinical/pathological factors alone to predict recurrence and progression, and an inability to predict response to systemic therapies and radiotherapy. The consequence is that many patients are either undertreated, overtreated, or given therapies that are unlikely to benefit the patient.

Understanding the molecular pathology and biology of BCa could be useful to improve patients' stratification and decision-making. Recently, several reports have focused their attention on molecular biomarkers as

diagnostic and prognostic tools in MIBC, although their application in clinical practice remains, to date, unclear.

Prediction of disease stage at radical cystectomy

An accurate prediction of disease stage at diagnosis is of fundamental importance to risk-stratification and to select patients for neoadjuvant systemic therapies. Understaging disease after initial TURBT is over 40% despite examination under anesthesia and cross sectional imaging[36]. As such improving staging at diagnosis is important to appropriately treat patients. Several tissue-based biomarkers have been investigated for this purpose and have been integrated into predictive models. Mitra et al. firstly developed a pre-cystectomy decision model to predict pathological upstaging and oncological outcomes in cT2 patients undergoing RC[37]. This model was based on clinicopathologic variables such as the preoperative presence of hydronephrosis, evidence of deep muscolaris propria invasion and LVI as well as tumor growth pattern and count. Subsequently, Shariat et al. tested the accuracy of a preoperative panel of tissue-based biomarkers (p53, p21, p27, Ki67, and cyclin E1); the number of altered biomarkers was able to predict T-stage upstaging but not T- and/or N-stage upstaging; however, the accuracy of the model in the prediction of the T-stage upstaging was low (62%)[38]. Recently, a genomic subtyping classifier was used to evaluate pathological upstaging in a multi-institutional cohort of patients with cT1-T2 BCa treated with RC[39]. Luminal tumors showed a lower rate of upstaging to non-organ confined disease compared to non-luminal ones (34% versus 51%). Pending external validation, molecular characterization promises to transform the paradigm of BCa risk-stratification, thus paving the way to an even more personalized approach.

Prediction of oncological outcomes after radical cystectomy alone

There has been an interest in predicting the likelihood of recurrence in patients who underwent RC alone. These patients may benefit from adjuvant therapies such as chemotherapy[40] and multiple trials are evaluating the value of adjuvant checkpoint inhibitors. Key biomarkers evaluated for prediction of oncological outcomes are listed in Table 2.

Currently, p53 is the most studied prognostic biomarker in patients treated with RC, with conflicting results reported in the literature. In patients with BCa confined to the bladder, p53 has been associated with progression and survival, independently of tumor grade, stage, and lymph node status[41].

Human epidermal growth factor receptor 2 (HER2) is a tyrosine kinase transmembrane receptor involved in cycle cell regulation and cell proliferation. Its overexpression was associated with adverse pathological features at RC but its relationship with long-term oncological outcomes remains controversial[42,43].

The retinoblastoma protein (RB1) is a tumor suppressor gene, which acts as a negative regulator of cell cycle progression and has been proved to be dysregulated in several cancers. The loss of RB1 expression is an adverse prognostic biomarker in MIBC[44,45]. Inactivating RB1 mutation results in a lower expression of FGFR3 levels and is associated with worse cancer-specific mortality (CSM)[46].

Survivin, an inhibitor of apoptosis, was found to be associated with disease recurrence (HR 1.7, P = 0.04), CSM (HR 1.7, $P = 0.03$), and all-cause mortality (HR 1.7, $P = 0.04$) in 222 consecutive patients treated with RC after accounting for the effects of standard prognosticators[47]. These results have been externally validated and survivin status has been incorporated in a nomogram for the prediction of outcomes in patients with pT1-3N0M0 disease: the addition of survivin improved the accuracy of the model over standard clinicopathologic features for prediction of disease recurrence and CSM.

Despite these findings, the role of these biomarkers in clinical practice as single markers remains limited, mainly due to their unsatisfactory accuracy. Models based on the assessment of multiple markers (p53, p21, RB, cyclin E1, and p27) showed higher predictive accuracy compared to those based on single markers [48–50]. A prospective study of 216 patients treated with RC who underwent immunohistochemical staining for p53, p21, p27, cyclin E1, and Ki-67 found that in a multivariable model adjusting for the effects of standard prognosticators, only LVI and number of altered biomarkers were independent predictors of recurrence and CSM[51].

As already before mentioned, BCa molecular characterization is acquiring increasing importance in the prediction of prognosis in patients with MIBC. Recently, a consensus classification has been provided. Compared to luminal papillary tumors that were taken as reference, luminal non-specified and stroma-rich tumors showed similar outcomes while luminal unstable, basal/squamous, and neuroendocrine-like subtypes were associated with worse survival, with the latter representing the class with the worst prognosis (HR 2.18, $P < 0.05$). Moreover, this classification, besides providing a promising tool for risk-stratification, suggests possible therapeutic implications such as those related to targeted therapies, thereby representing a milestone for MIBC classification.

TABLE 2.

Prognostic biomarkers after radical cystectomy alone in muscle-invasive bladder cancer

Molecular pathway	Biomarker(s)	Method	n	Results	Year	Reference
Cell cycle regulation	Rb	IHC	38	Association with cancer-specific mortality	2012	45
	P21	IHC	692	Association with disease recurrence and cancer-specific mortality	2010	111
Cell death pathways	p53	IHC	692	Association with disease recurrence and cancer-specific mortality	2010	112
		IHC	243	Association with disease recurrence and cancer-specific mortality	1994	41
	erbB2 (HER2)	IHC	354	No association with oncological outcomes	2010	42
		IHC	198	Association with disease recurrence and cancer-specific mortality	2016	43
	Survivin	IHC	222	Association with disease recurrence and overall mortality	2007	47
Angiogenesis markers	VEGF	IHC	286	Association with cancer-specific mortality	2007	113
Markers of tumor cell invasion	E-Cadherin	IHC	25	Association with survival	1993	114
	MMPs	IHC	54	Association with disease recurrence	2003	115
Molecular markers	Luminal unstable	Transcriptome analysis	1750	Association with worse survival compared to luminal papillary subtype	2019	61
	Basal/squamous					
	Neuroendocrine-like					

IHC: immunohistochemistry

Prediction of response to neoadjuvant chemotherapy

Key biomarkers investigated for prediction of response to NAC are listed in Table 3.

Cisplatin acts as an alkylating agent and interferes with DNA replication and gene transcription. This DNA-damage is repaired by 2 pathways: the first includes BRCA1, BRCA2, and RADS51 genes while the second involves the nucleotide excision repair NER, and includes several genes such as ERCC1-5, CDK7, DDB1-2, XPA. Alteration of these pathways has been suggested to affect the response to cisplatin-based chemotherapy. The breast cancer susceptibility gene 1 (BRCA1) modulates chemoresistance encoding a nuclear protein that responds to DNA damage with several different mechanisms. Patients with low/intermediate BRCA1 levels were found to have a significantly higher pathological response at RC compared to patients with high BRCA1 levels (66% versus 22%, $P = 0.01$)[52]. The excision repair cross-complementing 1 (ERCC1) is involved in DNA repair and DNA recombination: it was found associated with cisplatin resistance different tumors[53–55], whereas its role in BCa remains debated[56]. Genomic alterations in the DNA repair-associated genes *ATM*, *RB1*, and *FANCC* were found to be predictors of response (87% sensitivity, 100% specificity) and better OS after MVAC chemotherapy for

MIBC[57,58]. Regarding the ability of p53 mutation to predict response to NAC, conflicting results have been reported[58,59].

Recent studies evaluated the role of molecular profiles for decision-making and counseling of patients treated with NAC and RC. While there is some evidence that different tumor subtypes (basal, luminal, p53-like) are associated with different patterns of response to NAC [60], in the recently developed international consensus about the molecular classification of MIBC[61] no significant association between the consensus classes and oncologic outcomes in patients treated with NAC was found.

Prediction of response to systemic chemotherapy

Several cell cycle regulators and markers of proliferation have been evaluated as predictors of chemotherapy response[62,63]. Alongside this, a combination of regulatory RNAs and transcription factors has shown to be predictive in metastatic BCa patients treated with cisplatin-based therapy[64]. However, there is yet to be a clinically validated role for them as predictive markers.

The most promising class of predictive markers thus far for chemotherapeutic response is represented by those involved in DNA damage detection and repair (ie, BRCA-1, BRCA-2, RAD51, PAR, PARP1, ERCC1, ERCC2, and RRM1). In a study of patients with advanced or metastatic urothelial cancer receiving platinum-based palliative chemotherapy, 341 genes including 34 DNA damage response (DDR)-associated genes were evaluated[65]. Patients with DDR gene alterations had significantly longer progression-free and OS than patients with wild-type DDR genes. In the setting of advanced urothelial carcinoma, overexpression of ERCC1, RAD51, and PAR has been correlated with worse survival for patients treated with first-line platinum combination chemotherapy[66,67].

Aberrations of growth factors and their associated tyrosine kinase receptors can result in an abnormal increase in the rate of transduction of growth signals, thereby leading to uncontrolled cellular proliferation and tumor formation. Such kinases are the targets of several new systemic therapies in oncology. Several tyrosine kinase inhibitors have been tried in BCa including lapatinib (inhibits EGFR and HER2/neu pathways), and pazopanib (inhibits FGF, PDGF, and VEGF pathways). While these drugs appear to have limited activity in BCa, the possibility of biomarker enrichment for response has been assessed with mixed results[68–72].

TABLE 3.
Biomarkers associated with response to neoadjuvant chemotherapy

Molecular pathway	Biomarker(s)	Method	Drug(s)	n	Results	Year	Reference
Cellular efflux	CTR1	IHC	Cis	47	Association with pathologic response	2016	116
Cell death pathways	p53	IHC	MVAC	111	Association with survival	1995	59
		IHC	MVAC	44	Does not predict pathologic response	2014	58
	Bcl-2	IHC	Cis + radiotherapy	51	Low levels associated with better prognosis	2000	117
DNA repair	BRCA-1	rtPCR	Cis	57	Low/intermediate levels predict pathologic response	2011	52
	ERCC1	IHC	Cis	38	Does not predict pathologic response but predicts survival	2013	118
	ERCC2	rtPCR	Cis	50	Predicts pathologic response	2014	119
Molecular markers	Basal subtype	Transcriptome analysis	Cis	343	Association with better survival	2019	120

MVAC: methotrexate, vinblastine, doxorubicin, cisplatin, Cis: cisplatin-based, IHC: immunohistochemistry

Several other factors have been assessed for their ability to predict chemotherapy response, including immunological markers[73], germline and somatic DNA mutations[74,75], as well as drug transport genes[62,76]. Several of these factors are summarized in Table 4.

Prediction of response to systemic immunotherapy

The advent of systemic immunotherapy in the management of advanced BCa represents a quantum leap over the last few years, especially in patients refractory to cisplatin-based therapies. Several immune checkpoint inhibitors have shown promising activity, including agents targeting PD-1 receptor and its ligand PD-L1, and cytotoxic T-lymphocyte antigen 4 (CTLA-4). While these developments are promising, a majority of patients still do not respond to treatment[77–84], resulting in a significant financial burden and potential treatment-related side effects. This highlights the need for appropriate biomarkers to aid in selecting patients who are most likely to benefit from checkpoint targeting therapy. While several biomarkers have been explored

TABLE 4.

Biomarkers associated with systemic chemotherapy and immunotherapy response

Molecular pathway	Biomarker(s)	Method	Drug(s)	n	Results	Year	Reference
Cell cycle and proliferation	Cyclin D1	IHC	Cis	63	Overexpression predicts better chemo response	2016	63
	CCDN1	FISH	Cis	63	Does not predict chemo response	2016	63
	Ki-67	IHC	CMV, MVAC	99	Does not predict chemo response	1998	62
	miRNAs	rtPCR	MVAC, GC	83	Increased miR-21, miR-372 and E2F1 associated with chemo response and survival	2013	64
Cell death pathways	p53	IHC	MC, MEC CMV, MVAC	83	Overexpression predicts improved survival in chemoresistant patients	1999	121
		IHC	CMV, MVAC	99	Does not predict chemo response	1998	62
		IHC	CISCA, MVAC	25	Overexpression associated with worse response	1998	122
		IHC	MVAC	114	Does not predict chemo response (phase III RCT)	2011	123
	Bcl-2	IHC	Cis	51	Low expression predicts better response to chemoradiation	2000	117
		IHC	CISCA, MVAC	25	Overexpression associated with worse response	1998	122

MVAC: methotrexate, vinblastine, doxorubicin, cisplatin, MC: methotrexate, cisplatin, CMV: cisplatin, methotrexate, vinblastine
MVEC: methotrexate, vinblastine, epirubicin, cisplatin, MEC: methotrexate, epirubicin, cisplatin, GC: gemcitabine, cisplatin
GCT: gemcitabine, cisplatin, paclitaxel, Cis: cisplatin, CISCA: cisplatin, doxorubicin, cyclophosphamide, IHC: immunohistochemistry

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TABLE 4.
Biomarkers associated with systemic chemotherapy and immunotherapy response, *Cont'd*

Molecular pathway	Biomarker(s)	Method	Drug(s)	n	Results	Year	Reference
DNA repair	BRCA-1	IHC	Cis	104	Does not predict chemo response	2015	66
		rtPCR	GC, GCT	57	Does not predict chemo response	2007	67
	BRCA-2	IHC	Cis	104	Does not predict chemo response	2015	66
	RAD51	IHC	Cis	104	Overexpression associated with worse survival	2015	66
	PAR	IHC	Cis	104	Overexpression associated with worse survival	2015	66
	PARP1	IHC	Cis	104	Does not predict chemo response	2015	66
	ERCC1	IHC	Cis	104	Overexpression associated with worse survival	2015	66
		rtPCR	GC, GCT	57	Overexpression associated with worse survival	2007	67
	RRM1	rtPCR	GC, GCT	57	Does not predict chemo response	2007	67
Drug resistance	MDR1	rtPCR	MVEC	108	Overexpression associated with inferior outcome	2010	76
	P-glycoprotein (MDR1)	IHC	CMV, MVAC	99	Does not predict chemo response	1998	62
	Caveolin-1	rtPCR	GC, GCT	57	Does not predict chemo response	2007	67

MVAC: methotrexate, vinblastine, doxorubicin, cisplatin, MC: methotrexate, cisplatin, CMV: cisplatin, methotrexate, vinblastine
MVEC: methotrexate, vinblastine, epirubicin, cisplatin, MEC: methotrexate, epirubicin, cisplatin, GC: gemcitabine, cisplatin
GCT: gemcitabine, cisplatin, paclitaxel, Cis: cisplatin, CISCA: cisplatin, doxorubicin, cyclophosphamide, IHC: immunohistochemistry

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in the context of these clinical trials, no pretreatment recommendations can be definitively made at this point based on any molecular predictors, since a significant proportion of patients do respond to treatment despite testing negative for a biomarker. Nevertheless, biomarker-based selection for immunotherapy remains an area of active interest that is likely to further develop in the years to come.

PD-L1 expression has been associated with higher tumor grade, worse outcomes, and decreased postoperative survival[85,86]. In the IMvigor210 trial, a higher PD-L1 expression score was associated with a higher response rate[77]. In contrast, the CheckMate 275 trial showed meaningful responses to nivolumab, irrespective of PD-L1 expression levels[78]. Lack of standardized testing and evaluation of PD-L1 may be partially responsible for these discrepancies. Additionally, PD-L1 expression has been variously assessed: on tumor-infiltrating immune cells in the IMvigor210 trial[77], on tumor cells in the CheckMate 275 trial[78], and on both tumor cells and immune cells in the durvalumab trial[84]. Furthermore, there are variations in percentage cutoffs used to define the high and low expression. Finally, PD-L1 expression is

TABLE 4.
Biomarkers associated with systemic chemotherapy and immunotherapy response, *Cont'd*

Molecular pathway	Biomarker(s)	Method	Drug(s)	n	Results	Year	Reference
Cell growth signaling	FGFR3	WES	Pazopanib	3	Mutation associated with partial response	2016	68
	erbB2 (HER2)	WES	Pazopanib	3	Mutation associated with better response	2016	68
		IHC	Lapatinib	116	Does not predict chemo response	2017	69
		IHC	Lapatinib	34	Does not predict chemo response	2009	70
	HER1	IHC	Lapatinib	116	Does not predict chemo response	2017	69
	EGFR	IHC	Lapatinib	34	Overexpression associated with response	2009	70
	VEGF	Serum	Sunitinib	26	Does not predict chemo response	2014	71
		Serum, IHC	Pazopanib	18	Does not predict chemo response	2013	72
	HIF1α	IHC	Pazopanib	18	Does not predict chemo response	2013	72
DNA markers	Germline SNPs	Microarray	Cabazitaxel	45	SNPs predicted chemo response and toxicity	2016	74
		Microarray	Cis	210	SNPs predicted chemo response	2013	75
Immune markers	IL-8	Luminex xMAP	Sunitinib	38	Underexpression associated with better time to progression	2011	73
Other	Metallothionein	IHC	CMV, MVAC	99	Overexpression associated with worse survival	1998	62

MVAC: methotrexate, vinblastine, doxorubicin, cisplatin, MC: methotrexate, cisplatin, CMV: cisplatin, methotrexate, vinblastine
MVEC: methotrexate, vinblastine, epirubicin, cisplatin, MEC: methotrexate, epirubicin, cisplatin, GC: gemcitabine, cisplatin
GCT: gemcitabine, cisplatin, paclitaxel, Cis: cisplatin, CISCA: cisplatin, doxorubicin, cyclophosphamide, IHC: immunohistochemistry

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dynamic, and a single biopsy is unlikely to provide a complete assessment of status for the entire duration of disease. Therefore, evaluation of the predictive value of PD-L1 positivity is difficult, and correlations with response to treatment or survival vary between trials.

Despite these discrepancies, both pembrolizumab and atezolizumab are approved by the FDA and the EMA for first-line treatment in cisplatin-ineligible patients only in case of positive PD-L1 status on the basis of unpublished results from ongoing phase II trials. Patients with negative PD-L1 expression should be treated with chemotherapy-based combinations.

Exploratory analyses from the cisplatin pre-treated arm of the IMvigor210 trial showed that molecular subtypes were independently associated with response to atezolizumab treatment[77]. PD-L1 immune cell prevalence was highly enriched in the basal subtype versus the luminal subtype (60% versus 23%, $P < 0.001$). Response to atezolizumab occurred in all subtypes but was significantly higher in luminal cluster II than in other subtypes[77]. Conversely, in CheckMate 275, the basal-1 subtype had the highest proportion of

TABLE 4.
Biomarkers associated with systemic chemotherapy and immunotherapy response, *Cont'd*

Molecular pathway	Biomarker(s)	Method	Drug(s)	n	Results	Year	Reference
Immune markers	PD-L1	IHC	Atezolizumab	310	Overexpression associated with increased response to systemic immunotherapy	2016	77
		IHC	Nivolumab	265	Does not predict response to systemic immunotherapy	2017	78
		IHC	Pembrolizumab	114	Overexpression associated with increased response to neoadjuvant immunotherapy	2020	86
Molecular markers	Luminal cluster II	Transcriptome analysis	Atezolizumab	310	Better response compared to other subtypes	2016	77
	Basal-1	Transcriptome analysis	Nivolumab	265	Better response compared to other subtypes	2017	78

MVAC: methotrexate, vinblastine, doxorubicin, cisplatin, MC: methotrexate, cisplatin, CMV: cisplatin, methotrexate, vinblastine
MVEC: methotrexate, vinblastine, epirubicin, cisplatin, MEC: methotrexate, epirubicin, cisplatin, GC: gemcitabine, cisplatin
GCT: gemcitabine, cisplatin, paclitaxel, Cis: cisplatin, CISCA: cisplatin, doxorubicin, cyclophosphamide, IHC: immunohistochemistry

responders[78]. These discrepancies may be partially attributable to the fact that both trials allowed biopsy specimens from the primary tumor, lymph nodes, or metastatic lesions for subtyping, which may lead to inaccurate tumor classification. Until further details emerge, molecular classification may not be a reproducible predictive biomarker for immunotherapy.

The role of neoadjuvant therapy with checkpoint inhibitors is also gaining interest. The PURE-01 study evaluated the activity of preoperative pembrolizumab (NCT02736266) administered in T2-4aN0M0 MIBC patients[87]. In total, 114 patients were enrolled, and the pT0 rate was 37% (95% CI 28 to 46) while pT ≤ 1 rate was 55% (95% CI 46 to 65). On multivariable analysis, tumor mutational burden and PD-L1 combined positive score were associated with both the pT0 and the pT ≤ 1 response, regardless of tumor histology. A separate study using RNA sequencing found that the Immune190 signature was significant for complete response on multivariable logistic regression analyses in PURE-01, but not in a cohort of patients who underwent NAC and RC[88]. Hallmark signatures for interferon gamma (IFNγ; OR 1.11, $P = 0.004$) and IFNα response (OR 1.07, $P = 0.006$) were also associated with complete response for PURE-01, but not for NAC (IFNγ: OR 0.99, $P = 0.9$ and IFNα: OR 0.99, $P = 0.8$). Basal subtypes (across classifications) with higher Immune190 scores showed 100% 2-year progression-free survival after pembrolizumab therapy.

High mutational load may be associated with better response to immunotherapy[77]. However, there is currently no standardized definition of mutation burden relative to the depth of sequencing performed. Targeted sequencing panels may also not adequately cover gene fusions, truncations, and translocations. Further, germline variants may not be silenced by informatics techniques that filter common germline single-nucleotide polymorphisms. These challenges currently limit the use of tumor mutational burden as a predictive biomarker for immunotherapy.

Finally, the tumor microenvironment may play a role in predicting response to therapy[89]. CheckMate 275 found that the highest CXCL9 or CXCL10 expression was observed in nivolumab responders[78]. The same findings were reported by analyzing the cohort of cisplatin-pretreated patients of the IMvigor210 trial[77]. Immune markers investigated for the prediction of response to systemic immunotherapy are summarized in Table 4.

TABLE 5.
Biomarkers associated with radiotherapy response

Molecular pathway	Biomarker(s)	Chemoradiation regimen	n	Results	Year	Reference
Cell proliferation	Ki-67	RT 59.4 Gy + cisplatin	70	Higher Ki-67 associated with higher CR	2000	124
		RT 40 Gy + cisplatin	94	Higher Ki-67 associated with higher CR	2015	125
		RT 40.5 Gy (median) + cisplatin	62	No association with response	2004	126
Cell death pathways	Apoptotic index	RT 59.4 Gy + cisplatin	70	Higher index associated with higher CR	2000	124
	Bax/Bcl-2 ratio	RT 40.5 Gy (median) + cisplatin	62	Higher ratio associated with higher CR	2004	126
DNA repair	ERCC1	RT 40-66 Gy + cisplatin or nedaplatin	22	Expression loss associated with higher CR	2011	127
	ERCC1, XRCC1	RT 48.6 Gy (median) + cisplatin	157	Positive expression associated with improved survival	2013	128
	MRE11	RT 55 Gy	179	High expression associated with improved survival	2010	129
	DDR alterations	RT or chemoradiation	48	Presence of alterations associated with trend to improved recurrence-free survival	2016	130
Cell growth signaling	erbB2	RT 40 Gy + cisplatin + other agents	55	Positivity associated with lower CR	2005	131
		RT 40 Gy + cisplatin	119	Positivity associated with lower CR	2014	132
		RT 64.8 Gy + paclitaxel with (group 1: erbB2+) or without trastuzumab (group 2: erbB2-)	66	CR rates, 72% for group 1 and 68% for group 2	2017	133
Other	Molecular subtype	RT 40 Gy + cisplatin	118	CR rates, 52%/45%/15% for GU/SCC-like/ urobasal	2018	134
	Hsp60	RT 40 Gy + cisplatin	54	Positivity associated with better response	2007	135

RT: radiotherapy, CR: complete response, DDR: DNA damage response, GU: genomically unstable, SCC: squamous cell cancer

Prediction of response to radiotherapy

In the modern era, radiotherapy is generally administered in the context of organ preservation therapy in BCa. With careful patient selection, TMT yields oncological outcomes and quality of life comparable to RC in MIBC[90–92]. However, those who do not achieve complete response may undergo salvage cystectomy, with unfavorable oncological outcomes[93,94]. It is therefore imperative to carefully select patients who may be the optimal candidates for TMT. Several studies have looked at biomarkers that can predict response to TMT; the logic for evaluation of these biomarkers is generally based on their ability to predict response to the radiotherapy aspect of TMT.

Several different biomarkers such as cellular proliferation markers (ie, Ki-67), cell cycle regulators (ie, p53, Bcl-2, Bax, Bad), cell growth factors (ie, HER2/neu), and DNA damage repairs genes have been studied for this purpose. However, because of conflicting results between studies or lack of external validations, none of these markers is currently available for clinical practice. Important biomarkers that are predictive of response to radiotherapy in the context of TMT are listed in Table 5.

Tissue-based biomarkers and target therapies

Biomarkers are important not only because of their ability to predict outcomes or response to therapy but also, and more importantly, because they could act as potential targets for biomarkers-directed therapies.

This is the case of erdafitinib, the first FDA-approved oral pan-fibroblast growth factor receptor (FGFR) kinase inhibitor that binds to 4 FGFRs (FGFR-1 to -4), leading to decreased cell signaling and cellular apoptosis. The efficacy of erdafitinib (Balversa) has been tested in 99 patients with advanced or metastatic urothelial cancer progressing after at least one cycle of chemotherapy with FGFR2 or FGFR3 alterations[95]. The overall response was 40%, with 3% of patients experiencing complete response and 37% experiencing partial response. Of note, response was observed also in patients previously treated with systemic immunotherapy (response rate of 59% in this subgroup of patients).

Following these promising results, FGFR3 inhibitors (eg, infigratinib) are currently under investigation in the adjuvant setting after RC (EudraCT 2019-003248-63).

Conclusions

Reviewing the literature on the utility of tissue-based biomarkers in BCa through the last 2 decades, it appears obvious that the focus of research has moved from immunohistochemical analysis and tumor-related phenotypic changes to the analysis of genetic alterations. Furthermore, a trend towards marker combinations and genetic classifiers, mostly combining these findings with clinical parameters, is observed.

In summary, the literature on the prediction of disease recurrence in NMIBC is inconclusive, and little information is available for prediction of response to intravesical BCG. Concerning disease progression, external prospective validation studies suggest that mutational FGFR3 status and gene signatures may improve models on the basis of clinicopathologic information.

In MIBC, tissue-based biomarkers are increasing their importance since they may predict the response to systemic chemotherapy and immunotherapy. The advent of molecular characterization carries the promise to revolutionize the paradigm of decision-making in the treatment of MIBC, especially in these years characterized by the advent of systemic immunotherapy.

Prospective studies in well-defined patient cohorts and with clinically meaningful endpoints are needed for retrieving definitive conclusions about the utility of tissue-based biomarkers in BCa. Until then, their role, despite their promising value, should be limited to experimental settings.

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