Molecular biomarkers in bladder preservation therapy for muscle-invasive bladder cancer

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Although muscle-invasive bladder cancer is commonly treated with radical cystectomy, a standard alternative is bladder preservation therapy, consisting of maximum transurethral bladder tumour resection followed by radiotherapy with concurrent chemotherapy. Although no successfully completed randomised comparisons are available, the two treatment paradigms seem to have similar long-term outcomes; however, clinicopathologic parameters can be insufficient to provide clear guidance in the selection of one treatment over the other. Recent advances in the molecular understanding of bladder cancer have led to the identification of new predictive biomarkers that ultimately might help guide the tailored selection of therapy on the basis of the intrinsic biology of the tumour. In this Review, we discuss the existing evidence for molecular alterations and genomic signatures as prognostic or predictive biomarkers for bladder preservation therapy. If validated in prospective clinical trials, such biomarkers could enable the identification of subgroups of patients who are more likely to benefit from one treatment over another, and guide the use of combination therapies that include other modalities, such as immunotherapy, which might act synergistically with radiotherapy.

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Introduction

Bladder cancer is the ninth most common cancer in the world, with an estimated 430 000 new diagnoses annually worldwide, resulting in 165 000 deaths per year.[1] Nearly a quarter of patients have muscle-invasive bladder cancer, which is characterised by a high propensity for rapid growth and metastasis if not managed with aggressive therapy. Although radical cystectomy is the most commonly recommended treatment for muscle-invasive bladder cancer, several single institution and cooperative group studies have shown that bladder-sparing trimodality therapy (TMT), typically comprised of a maximum transurethral resection of bladder tumour (TURBT) followed by radiotherapy with concurrent chemotherapy, results in long-term outcomes similar to those of radical cystectomy.[2–8] Pooled results from six reported Radiation Therapy Oncology Group (RTOG) phase 2 trials of bladder preservation for muscle-invasive bladder cancer showed that 69% patients achieved a complete response and the 5-year disease-specific survival was of 71%.[2] A randomised, multicentre, feasibility trial directly comparing radical cystectomy and bladder preservation therapy was not completed because of poor accrual.[9] However, a recent propensity score-matching analysis showed that radical cystectomy and bladder-sparing TMT have similar outcomes, with 5-year disease-specific survivals of 73·2% and 76·6% in the radical cystectomy and TMT cohorts, respectively.[8] Outcomes of bladder-sparing TMT in the modern era (2005–13) show that up to 88% patients can achieve a complete response and 5-year disease-specific survival can be as high as 84%, which compares favourably with contemporary radical cystectomy series.[4] Thus, bladder preservation therapy seems to be an acceptable alternative to radical cystectomy for appropriately selected patients with muscle-invasive bladder cancer and is often associated with excellent quality-of-life outcomes because of the retention of the patient's native bladder.[10] As a result, many national and international cooperative group consensus guidelines now recommend both radical cystectomy and bladder preservation therapy as effective treatment options for muscle-invasive bladder cancer, including the National Institute for Health and Care Excellence, the National Comprehensive Cancer Network, the European Association of Urology, the American Urological Association, the American Society of Clinical Oncology, the American Society for Radiation Oncology, and the Society of Urologic Oncology.[11–14]

Clinicopathological factors have an important role in the selection of patients suited for bladder preservation therapy. Variables known to be associated with survival outcomes after bladder-sparing therapy include tumour-related factors, such as T stage, the presence of hydronephrosis, and the presence of carcinoma in situ; and treatment-related factors, such as the completeness of TURBT and whether or not patients achieve a complete response to induction chemotherapy or chemoradiation.[3,4,15] However, many tumour-related features, including T stage and presence of hydronephrosis, are also prognostic factors in radical cystectomy cohorts. In some cases, clinical or social factors, such as medical comorbidities or strong patient preference, can direct patients towards one therapy over another. Nonetheless, standard clinicopathological features are often insufficient to accurately predict outcomes or guide therapy choice. Therefore, the identification of molecular biomarkers that can predict therapeutic outcomes with greater precision, and ultimately inform the appropriate selection of therapy for each patient, is an urgent clinical need.

Our rapidly growing understanding of the molecular and genomic characteristics of bladder cancer through next-generation sequencing and gene expression profiling has led to the identification of new potential molecular biomarkers for muscle-invasive bladder cancer. These biomarkers can be categorised broadly as either prognostic or predictive factors. Prognostic biomarkers are associated with differing clinical

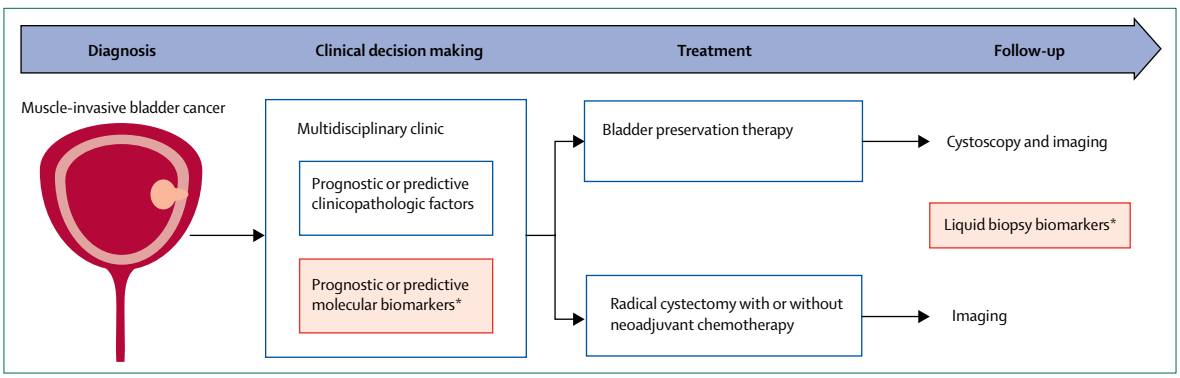


Figure 1: **Schematic of the decision-making workflow for the clinical management of muscle-invasive bladder cancer, guided by prognostic and predictive molecular biomarkers**

*Biomarkers could be used to aid decision making and during treatment follow-up.

outcomes based on the biology of the cancer and the patient. Predictive biomarkers are used to identify subgroups of patients who are likely to benefit from specific therapies and indicate their probability of response to a particular treatment. Both prognostic and predictive molecular biomarkers can be of great value in providing rational guidance for the tailored selection of therapy on the basis of molecular characteristics of the patient's tumour (figure 1, table). In this Review, we discuss the current evidence regarding prognostic and predictive biomarkers in bladder preservation therapy for muscle-invasive bladder cancer.

DNA repair pathway alterations

Most bladder tumours show a complex genetic landscape characterised by a high point mutation burden and frequent copy number alterations and chromosomal translocations.[28] These genomic alterations are thought to be driven by chronic exposure of the urothelium to genotoxic agents, such as tobacco byproducts and other environmental agents (including aromatic amines) that have been linked to bladder cancer risk.[29] However, several emerging lines of evidence show that bladder tumours often also have alterations in DNA repair pathway genes, and functional DNA repair deficiency can contribute to genomic instability and drive bladder tumour evolution and response to treatment.[30] Therefore, the scientific community now has a widespread interest in identifying DNA repair biomarkers that could help oncologists to make decisions regarding treatment options for bladder cancer (figure 2).

One of the first DNA repair biomarkers to be investigated as a biomarker of radiotherapy response in patients with muscle-invasive bladder cancer was MRE11. MRE11 is a DNA nuclease that has a crucial role in response to DNA damage, including double-strand breaks and stalled replication forks.[31] MRE11 interacts dynamically with binding partners (eg, NBS1 and RAD50) to coordinate DNA processing at damage sites and maintain genomic integrity. Choudhury and colleagues[16] used an immunohistochemistry approach to measure the amount of MRE11 and several other double strand break-related proteins in pretreatment tumour biopsies from a cohort of patients with muscle-invasive bladder cancer managed with radical radiotherapy alone. MRE11 was the only protein with a degree of immunohistochemistry staining that was associated with radiotherapy outcomes. In a test cohort of 86 patients, individuals with an amount of tumour MRE11 protein in the lowest quartile had significantly worse 3-year cancer-specific survival than patients whose tumours had higher MRE11 (cancer-specific survival 43% *vs* 69%, p=0·012), and this association was validated in an independent cohort of 93 patients (43% *vs* 71%, p=0·020). However, no association was found between MRE11 staining signal and outcomes in a cystectomy cohort (62% *vs* 54%, p=0·46), suggesting that the amount of MRE11 might be predictive of radiotherapy response, but not prognostic in the absence of radiotherapy. A similar association between low MRE11 staining and decreased response to radiotherapy has now also been reported by other groups. Laurberg and colleagues[17] showed that low MRE11 staining was associated with worse disease-specific survival in a cohort of 148 patients treated with bladder preservation therapy (83% of whom received concurrent chemotherapy), but found no association between MRE11 and outcomes in a cohort of 273 patients treated with cystectomy.[17] In a cohort of 135 patients with muscle-invasive bladder cancer from six RTOG phase 2 trials treated with bladder-sparing TMT, MRE11 expression was quantified by calculating the nuclear to cytoplasmic MRE11 staining ratio, and patients with an MRE11 nuclear-to-cytoplasmic ratio in the lowest quartile (ratio ≤1·49) had a 4-year disease-specific mortality of 41% compared with 21% for patients with a ratio higher than 1·49.[18] Notably, in contrast to the studies by Choudhury and colleagues and Laurberg and colleagues, which used proprietary research-grade approaches with quartiles instead of absolute measurements, the RTOG group developed an automated and standardised approach to detect and quantify MRE11 expression with thresholds based on absolute expression, which is eligible

		Prognostic or predictive	Method	Patients, n	Clinical correlation	Results
DNA repair genes						
MRE11	Choudhury et al (2010)[16]	Predictive	Immunohistochemistry	181 in RT group, 88 in RC group	RT: low *MRE11* expression had lower cancer-specific survival; RC: *MRE11* not associated with cancer-specific survival	RT: HR 0·39 (95% CI 0·23–0·66), p<0·001; RC: HR 1·50 (95% CI 0·70–3·20), p=0.29*
MRE11	Laurberg et al (2012)[17]	Predictive	Immunohistochemistry	148 in CRT group, 273 in RC group	CRT: low *MRE11* expression had lower disease-specific survival; RC: *MRE11* not correlated with disease-specific survival	CRT: HR 0·64 (95% CI 0·47–0·86), p=0·005; RC: HR 1·01 (95% CI 0·78–1·29), p=0.96*
MRE11	Magliocco et al (2017)[18]	Prognostic	Immunohistochemistry (nuclear/cytoplasmic ratio)	135, all had TMT	TMT: low *MRE11* nuclear/cytoplasmic ratio associated with higher disease-specific mortality	HR 2·0 (95% CI 1·1–3·8), p=0·03
MRE11	Teo et al (2014)[19]	Predictive	Targeted next-generation sequencing (germline MRE11A SNP)	256 in RC group, 186 in RT group	RC: *MRE11A* SNP rs1805363 not associated with cancer-specific survival; RT: *MRE11A* SNP rs1805363 associated with lower cancer-specific survival	RC: HR 0·99 (95% CI 0·61–1·60), p=0.89*; RT: HR 2·10 (95% CI 1·34–3·28), p=0·001
ERCC1	Sakano et al (2013)[20]	Prognostic	Immunohistochemistry	157, all had TMT	TMT: higher *ERCC1* and *XRCC1* associated with improved disease-specific survival	HR 0·64 (95% CI 0·43–0·95), p=0·024
ERCC2	Desai et al (2016)[21]	Prognostic	Next-generation sequencing	48, all had CRT	CRT: *ERCC2* mutations associated with less distant metastasis	0% *vs* 43% 2-year distant metastasis, p=0·044
Signal transduction genes						
EGFR	Chakravarti et al (2005)[22]	Prognostic	Immunohistochemistry	73, all had TMT	TMT: EGFR positivity associated with improved disease-specific survival, and HER2 positivity associated with reduced proportion of patients achieving a complete response	HR 0·12 (95% CI 0·02–0·93), p=0·042; complete response: 50% *vs* 81%, HR 4·25 (95% CI 1·19–15·14), p=0·026
HER2	Inoue et al (2014)[23]	Prognostic	Immunohistochemistry	119, all had CRT	CRT: high HER2 expression associated with shorter cancer-specific survival	HR 2·5 (95%CI not provided), p=0·025
VEGF-B	Lautenschlaeger et al (2014)[24]	Prognostic	Immunohistochemistry	43, all had TMT	TMT: high VEGF-B associated with lower overall survival	HR 2·83 (95% CI 1·22–6·59), p=0·01 (VEGF-B)
Immune checkpoint biomarkers						
PD-L1	Wu et al (2016)[25]	Prognostic	Immunohistochemistry	72, all had TMT	TMT: PD-L1 positivity associated with high local-regional failure	OR 0·093 (95% CI 0·029–0·301), p<0·001
Molecular signatures						
Hypoxia signature	Yang et al (2017)[26]	Predictive	RNA expression microarray (24-gene signature)	75 in RT group, 76 in RT + CON group	RT: hypoxia signature (more hypoxic) correlated with lower local relapse-free survival; RT + CON: hypoxia signature predicted benefit in local relapse-free survival from addition of CON to RT	RT: HR 2·37 (95% CI 1·26–4·47), p=0·0076; RT + CON: HR 0·47 (95% CI 0·26–0·86), p=0·015
Immunological signature	Miyamoto et al (2018)[27]	Predictive	RNA expression microarray	136 in TMT group, 223 in NAC + RC group	TMT: increased immune signature associated with improved disease-specific survival; NAC + RC: immune signature not associated with disease-specific survival	TMT: HR 0·30 (0·14–0·65), p=0·002; NAC + RT: p=0.063* (HR not provided)

RT=radiotherapy. HR=hazard ratio. RC=radical cystectomy. CRT=chemoradiation therapy. TMT=bladder-sparing trimodality therapy. SNP=single nucleotide polymorphism. PD-L1=programmed–death-ligand 1. OR=odds ratio. CON=carbogen and nicotinamide. NAC=neoadjuvant chemotherapy. *p>0·05 is not significant.

***Table*: Prognostic and predictive value of selected biomarkers in bladder preservation therapy for muscle-invasive bladder cancer**

for Clinical Laboratory Improvement Amendments (CLIA) approval and can potentially be used in the clinic.[18]

Overall, these data suggest that low MRE11 protein expression is associated with worse outcomes following radiotherapy-based treatment of muscle-invasive bladder cancer, and that MRE11 could serve as a predictive biomarker to guide treatment decisions for this type of cancer. The finding that low concentrations of a DNA repair protein, such as MRE11, are associated with worse outcomes is interesting, since low expression could be postulated to be associated with decreased DNA repair and increased sensitivity to the DNA-damaging effects of radiation. However, increased expression of DNA repair genes has been shown to be associated with increased sensitivity to DNA-damaging agents in other contexts. For example, the Recombination Proficiency Score[32] was developed to predict tumour sensitivity to DNA-damaging agents and is calculated based on the transcript levels of four DNA repair genes including *Rad51*, a central member of the homologous recombination pathway required for error-free repair of double strand-breaks.[32] High transcript concentrations result in a low Recombination Proficiency Score, which is associated with increased DNA damage sensitivity. Therefore, increased expression of an individual DNA repair protein (eg, MRE11) might represent a futile attempt by the tumour to upregulate DNA repair in a repair-deficient setting. Transcript amounts of the *MRE11A* gene were not associated with the level of MRE11

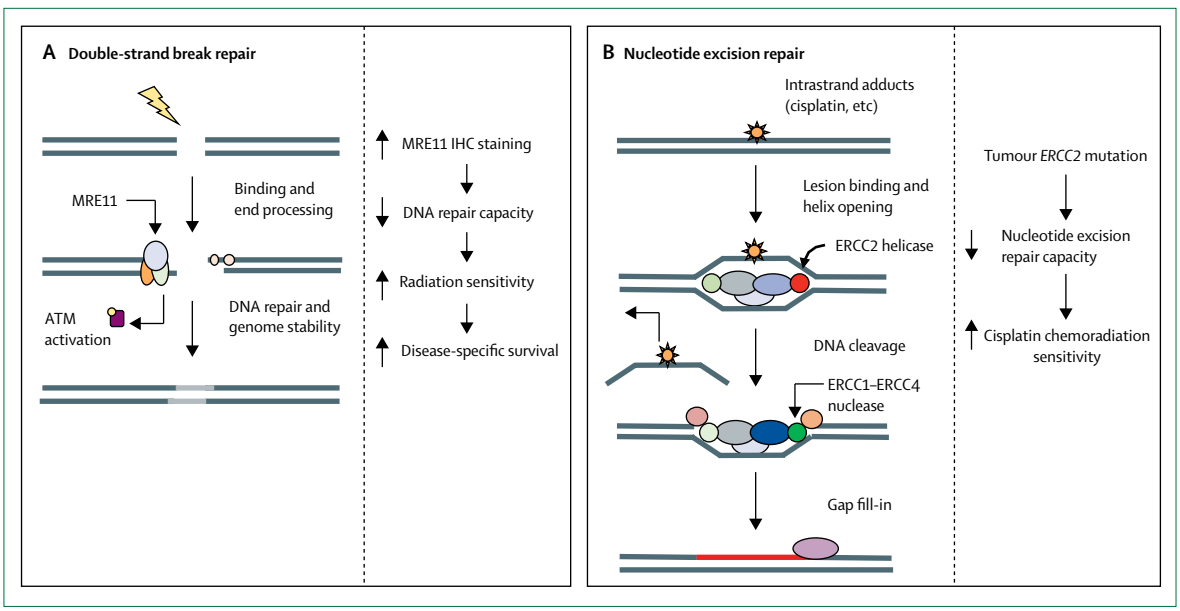


Figure 2: **DNA repair pathways that might be used as molecular biomarkers in bladder cancer**
(A) The double-strand break repair pathway. (B) The nucleotide excision repair pathway. IHC=immunohistochemistry. ATM=ataxia-telangiectasia mutated.

protein expression or clinical outcomes in one published study,[33] suggesting that control of MRE11 protein concentrations might mainly occur at the post-transcriptional level. In a separate study,[19] a germline single-nucleotide polymorphism in *MRE11A* was associated with worse outcomes in a radiotherapy-treated cohort of patients with muscle-invasive bladder cancer, but not in a cohort of patients treated with cystectomy. The presence of this single-nucleotide polymorphism was not associated with the amount of MRE11 protein measured by immunohistochemistry. The effect of germline events as predictors of tumour response in patients with muscle-invasive bladder cancer treated with bladder preservation therapy requires further investigation.

The associations between expression of other DNA repair proteins and muscle-invasive bladder cancer outcomes have also been investigated. However, most of these studies have been done in the setting of chemotherapy rather than radiotherapy. ERCC1 is a core member of the nucleotide excision repair pathway, a highly conserved DNA repair pathway responsible for repairing intrastrand lesions created by genotoxins such as ultraviolet radiation and platinum drugs.[34] In 2007, Bellmunt and colleagues[35] reported that low concentrations of ERCC1 mRNA, measured by RT-PCR, were associated with improved overall survival in patients with metastatic bladder cancer treated with platinum-based chemotherapy, supporting the hypothesis that decreased amounts of ERCC1 result in the loss of nucleotide excision repair capacity and increased platinum sensitivity. However, the opposite association has been observed in patients with muscle-invasive bladder cancer treated with radical cystectomy or TMT. In two studies, high expression of ERCC1 protein measured by immunohistochemistry was associated with improved cancer-specific survival outcomes.[20,36] In the TMT cohort, high expression of both ERCC1 and XRCC1 (a protein involved in repair of single-strand DNA breaks) was associated with improved disease-specific survival on multivariate analysis.[20] The effect of ERCC1 might vary across clinical contexts and treatment paradigms, and additional studies are needed to further define the role of DNA repair gene or protein expression as muscle-invasive bladder cancer biomarkers.

The widespread application of next-generation sequencing approaches has led to identification of frequent DNA repair pathway alterations in bladder tumours, and mutations in DNA repair genes have been associated with responses to cisplatin-based chemotherapy delivered in the neoadjuvant setting (before radical cystectomy) and in patients with metastatic disease.[37–40] Somatic missense mutations in the nucleotide excision repair helicase *ERCC2* were enriched in patients with muscle-invasive bladder cancer who achieved a pathologic complete response to neoadjuvant cisplatin-based chemotherapy before cystectomy in two independent cohorts,[41,42] and were associated with an overall survival benefit in these patients. Fewer genomic data are available for patients with muscle-invasive bladder cancer treated with radiotherapy-based approaches. However, in a cohort of 48 patients treated with TMT,[21] a predicted deleterious mutation in one or more DNA damage and response genes was associated with a trend towards reduced recurrence of bladder tumours and the subset of these patients with tumour *ERCC2* mutations also had significantly lower rates of metastatic recurrence than

those without these mutations (metastatic recurrence 0% *vs* 43%, p=0·044).

Alterations in signal transduction pathways

In addition to DNA repair pathways, cellular signal transduction pathways are also frequently altered in bladder cancer and might be used as potential biomarkers of treatment response and resistance. Several large-scale sequencing studies have shown that 35–55% of bladder tumours have mutations, translocations, or amplifications of signal transduction genes.[28,43] The ERBB family of receptor tyrosine kinases, of which EGFR was the first to be discovered, is altered by mutation, amplification, or overexpression in more than 30% of bladder tumours.[28,43] Expression of EGFR in muscle-invasive bladder cancer has been found to be associated with a favourable prognosis after bladder-sparing TMT. In a cohort of 73 patients enrolled in four prospective RTOG bladder preservation trials (RTOG 8802, 8903, 9506, and 9706) using cisplatin-containing chemoradiation, EGFR positivity assessed by immunohistochemistry was found to be significantly associated with improved overall survival (p=0·044), disease-specific survival (p=0·042), and disease-specific survival with intact bladder (p=0·021) on univariate analysis.[22] EGFR positivity remained significantly associated with improved disease-specific survival on multivariate analysis controlling for known clinical factors.[22] By contrast, immunohistochemistry positivity for HER2 expression in the same cohort of patients was significantly associated with reduced proportions of patients achieving a complete response after chemoradiation (complete responses 50% *vs* 81%, p=0·026). Similarly, Inoue and colleagues[23] reported that in a separate cohort of 119 patients with muscle-invasive bladder cancer who were treated with chemoradiation following TURBT, HER2 overexpression was an independent predictor of pathologic incomplete response to chemoradiotherapy (odds ratio 2·9; p=0·031) and shorter cancer-specific survival (5-year cancer-specific survival 56% *vs* 87%; p=0·001) on multivariate analysis. Therefore, expression of EGFR in muscle-invasive bladder cancer seems to be associated with a favourable prognosis after bladder-sparing TMT, whereas overexpression of HER2 is associated with resistance to chemoradiation treatment.

In the context of the poor prognosis and inferior complete response (to a variety of treatments including chemoradiation) associated with overexpression of HER2, biologic agents and small-molecule inhibitors that target HER2 have been tested in bladder cancer clinical trials in metastatic and localised disease settings. A multi-institutional phase 1–2 trial (RTOG number 0524)[44] was done to evaluate the addition of trastuzumab, an anti-HER2 monoclonal antibody, to chemoradiation with paclitaxel for HER2-overexpressing tumours in patients with muscle-invasive bladder cancer who were not candidates for cystectomy. However, because of a low proportion of HER2-positive patients in the study as measured by immunohistochemistry (only 20 [29%] of 68 enrolled patients were HER2 positive), the findings from the study were not conclusive regarding the efficacy of trastuzumab in the context of TMT.[44] Nevertheless, 13 (72%) of the 18 evaluable HER2-positive patients (a group generally considered to have poor outcomes) achieved a complete response at 1 year with this TMT regimen, which is a favourable result in comparison with historical controls (ie, HER-2 positive patients who received standard chemoradiation without trastuzumab), and warrants further study in additional biomarker-driven trials.

Angiogenesis

Angiogenesis has a crucial role in the growth and metastasis of a range of solid tumours, including bladder cancer. Elevated amounts of VEGF, a key angiogenesis signalling molecule, have been associated with increased disease recurrence and metastasis in muscle-invasive bladder cancer.[45] In a pooled analysis of 43 patients with muscle-invasive bladder cancer from four RTOG prospective bladder preservation studies,[24] VEGF expression was not associated with complete response after chemoradiation, but high concentrations of cytoplasmic VEGF-B, VEGF-C, and VEGF-R2 were associated with decreased overall survival (3-year overall survival: 43·7% [95% CI 20·5–64·8%] in patients with increased VEGF-B expression *vs* 75% [46·3–89·8%] in those with low VEGF-B expression, p=0·01; 40·2% [17·3%–62·2%] for high VEGF-C vs 86·7% [56·4%–96·5%] for low VEGF-C, p=0·01; 49·7% [25·4%–70·0%] for high VEGF-R2 vs 66·7% [40·4%–83·4%] for low VEGF-R2, p=0·02). Elevated VEGF-B was also associated with high rates of distant failure (3-year distant failure rate of 39·5% [95% CI 15·3%–63·7%] for high VEGF-B *vs* 22·3% [17·3%–27·2%] for low VEGF-B; p=0·01).[24] In another study, Keck and colleagues[46] reported on the prognostic value of expression of VEGF-C and its transmembrane glycoprotein receptor neuropilin-2 (NRP2) in a cohort of 247 patients with muscle-invasive bladder cancer treated with bladder preservation therapy. Elevated expression of both VEGF-C and NRP2, assessed by immunohistochemistry, were associated with short cancer-specific survival on univariate analysis (p=0·041 and p=0·037). Furthermore, co-expression of VEGF-C and NRP2 were predictive of shorter overall survival in multivariate models (hazard ratio [HR] 7·54 [95% CI 1·57%–36·23%], p=0·012). Therefore, increased expression of angiogenesis factors in the VEGF signalling axis is associated with poor prognosis after treatment of muscle-invasive bladder cancer with TMT, and might be able to identify patients who are more likely to benefit from early cytotoxic therapy or anti-VEGF targeted therapies. A recent phase 3 trial on the addition of the VEGF receptor 2 (VEGFR-2) antagonist ramucirumab to docetaxel in platinum-refractory advanced urothelial carcinoma resulted in superior progression-free survival

compared with docetaxel alone, validating VEGFR-2 signalling as a viable therapeutic target.[47] Whether or not a similar strategy of adding VEGFR-2 inhibitors to chemoradiation would improve the efficacy of bladder preservation therapy for tumours that overexpress VEGFR-2 is unknown.

Other tumour genetic features

Several additional proliferation and tumour suppressor genes have been investigated regarding their potential as candidate biomarkers in bladder preservation therapy, although the results regarding their prognostic value have been mixed. Rodel and colleagues[48] reported that high apoptotic index and Ki-67 expression were statistically significantly related to complete response, local control, and cancer-specific survival in a cohort of 70 patients with muscle-invasive bladder cancer treated with TMT. Similarly, high Ki-67 expression was associated with improved 5-year cancer-specific survival (78% in patients with high Ki-67 expression *vs* 46% in those with low Ki-67 expression, p=0·019) in a study of 94 patients with muscle-invasive bladder cancer treated with chemoradiation.[49] By contrast, a study by Laurberg and colleagues[17] showed that Ki-67 expression was not statistically significantly associated with disease-specific survival in a study of 148 patients with muscle-invasive bladder cancer treated with bladder preservation therapy. Thus, the prognostic value of Ki-67 staining in the setting of TMT remains unclear.

Similar mixed results have been noted with tumour suppressor gene modifications. Overexpression of p53 and p21 were reported as independent predictors of decreased disease-specific survival (p=0·005 for p53 overexpression and *vs* p=0·009 for p21 overexpression) in a study of 82 patients with muscle-invasive bladder cancer treated on three different TMT protocols.[50] However, the findings from studies by Rodel and colleagues,[48] Chakravarti and colleagues,[22] and Laurberg and colleagues[17] showed no significant association between p53 expression and clinical outcomes in patients treated with TMT. The study by Laurberg and co-workers did identify a significant association (HR 0·52 [95% CI 0·28–0·96], p=0·032) between p16 expression and disease-specific survival in the cohort of patients treated with radiotherapy, but the Chakravarti and co-workers study did not show any prognostic value for p16.[17,22] Another study showed that loss of the tumour suppressor gene *AIMP3* was associated with decreased overall survival in patients with muscle-invasive bladder cancer following radiotherapy, although these results await validation in further studies.[51] The conflicting data on the prognostic value of individual candidate biomarkers highlight the difficulties of generating robust and reproducible data through the retrospective analysis of small size cohorts. The validation of molecular biomarkers will, therefore, ultimately require prospective assessment in appropriately powered clinical trials.

Gene-expression signatures

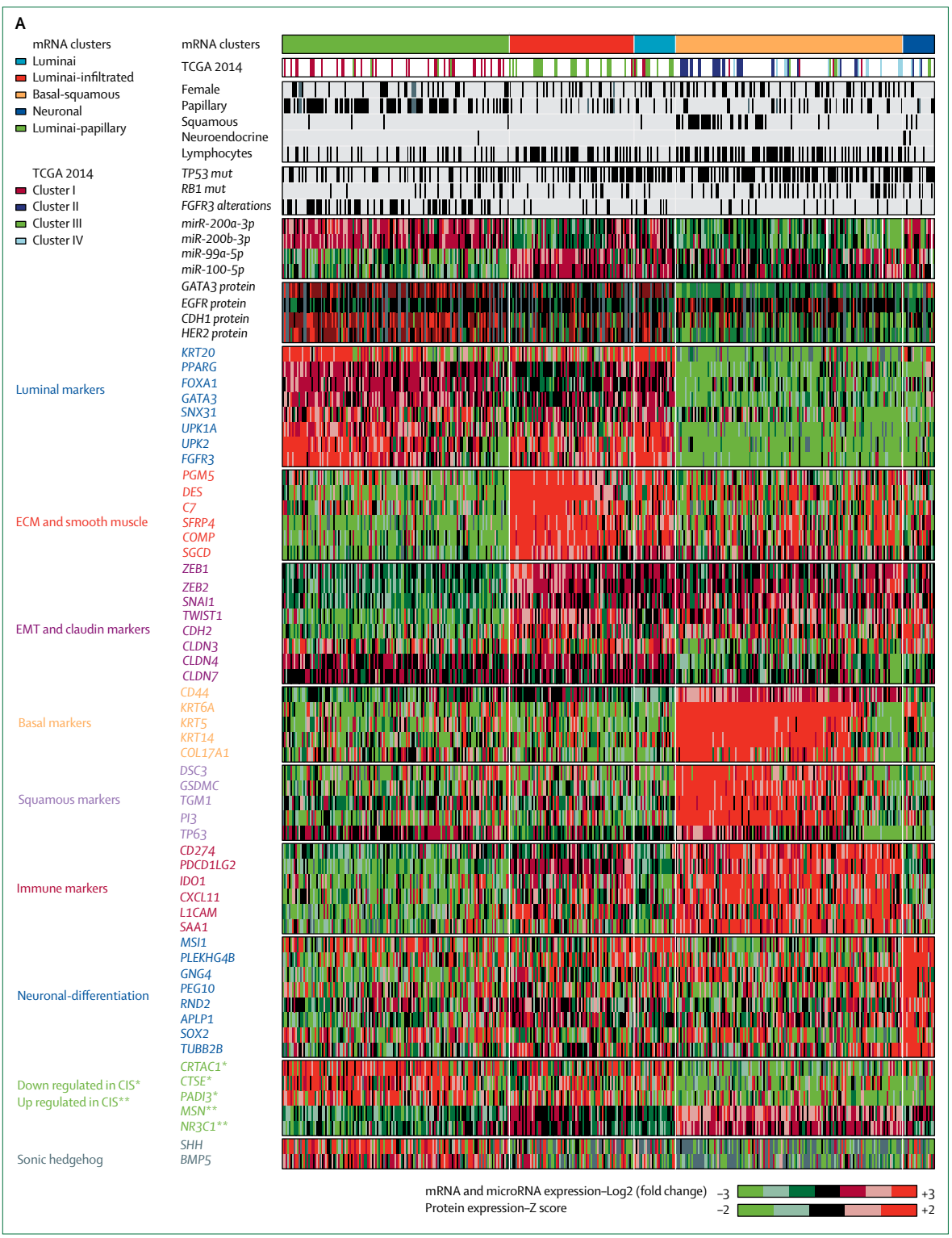
Recent genome-wide gene-expression profiling studies of bladder tumours have identified distinct biological subtypes, broadly categorised into luminal and basal subtypes, similarly to those found in breast cancer (figure 3A).[28,43,52–54] These gene expression-based molecular subtypes are associated with different prognoses after radical cystectomy, with basal tumours associated with short disease-specific survival and overall survival in patients undergoing radical cystectomy.[53,54] Patients with basal tumours have a substantial improvement in survival with the addition of neoadjuvant cisplatin-based chemotherapy to radical cystectomy compared with other subtypes of bladder tumours, suggesting that expression subtype is not only prognostic, but also predictive of response to treatment (figure 3B).[55,56] This variation in the benefit of DNA-damaging chemotherapy across molecular subtypes might be attributable to differences in DNA repair capacity between the various subtypes.[56] However, in the Cancer Genome Atlas cohort, somatic alterations in DNA repair genes were present across expression subtypes rather than enriched in a single subtype.[28,57] These alterations suggest that tumour DNA repair capacity might be regulated by genetic or epigenetic mechanisms beyond somatic mutations and that cellular features beyond DNA repair capacity might affect tumour response to neoadjuvant chemotherapy.[58]

The effect of molecular subtype in muscle-invasive bladder cancer on outcomes after bladder preservation therapy has not yet been clearly defined. In a series of 136 pre-TURBT resection specimens from patients who underwent bladder-sparing TMT at the Massachusetts General Hospital (Boston, MA, USA), the proportion of patients achieving a complete response, disease-specific survival, and overall survival were not significantly associated with molecular subtype in the TMT cohort.[27] Similar results by Yang and colleagues[59] indicated no significant difference in local relapse-free survival between bladder tumour subtypes in patients with muscle-invasive bladder cancer receiving radiotherapy alone. These results suggest that patients with basal muscle-invasive bladder cancer, which generally have a poor prognosis with radical cystectomy, might not have substantially worse outcomes compared with other subtypes when treated with radiotherapy-based bladder preservation therapy approaches. Analysis of other bladder preservation therapy cohorts will be needed to validate these findings.

Hypoxia

Many solid tumours have regions of hypoxia, due to tumour proliferation overtaking the ability of angiogenesis to meet an increasing demand for oxygen.[60] Hypoxia contributes to resistance to radiotherapy because of the important role of oxygen in the induction of DNA damage by ionising radiation.[60] Yang and colleagues[26] recently developed a 24-gene hypoxia signature and showed its prognostic and predictive value in the context of a phase 3

randomised trial of radiotherapy alone or with hypoxia modification consisting of carbogen and nicotinamide for muscle-invasive bladder cancer. The signature was found to be prognostic within the cohort of 75 patients receiving radiotherapy alone, with an HR of 2·37 (95% CI 1·26–4·47, p=0·0076) for local relapse-free survival. Furthermore,



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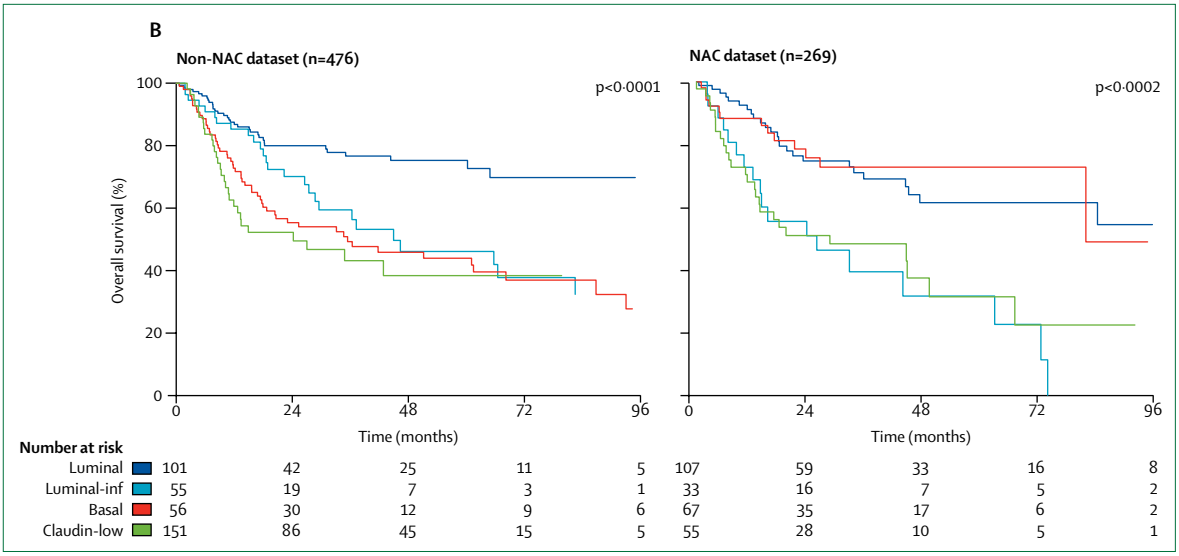

Figure 3: **Molecular subtypes of bladder cancer have prognostic and predictive value**
(A) mRNA expression subtypes from The Cancer Genome Atlas (TCGA). (B) Overall survival in non-neoadjuvant chemotherapy (NAC) cohorts (left) and NAC cohorts (right) stratified by molecular subtype. Luminal-inf=luminal-infiltrated. ECM=extracellular matrix. EMT=epithelial mesenchymal transition. CIS=carcinoma in situ. Adapted from Robertson et al,[28] by permission of Elsevier, and Seiler et al,[56] by permission of Elsevier.

the signature predicted a benefit from the addition of the hypoxia modification carbogen and nicotinamide to radiotherapy, with a HR of 0·47 (95% CI 0·26–0·86, p=0·015). The prognostic and predictive value of the hypoxia signature remained statistically significant after adjustment for clinicopathological variables. Thus, this signature might have prognostic value in patients with muscle-invasive bladder cancer undergoing bladder preservation therapy, and might also be used as a predictive biomarker to identify the subset of patients who would most benefit from the addition of hypoxia modification as part of their treatment regimen. However, the clinical value of carbogen or nicotinamide therapy will need to be evaluated in the context of chemoradiation therapy, since chemoradiation has been established as the standard of care over radiotherapy alone for muscle-invasive bladder cancer.[6]

Immunotherapy biomarkers

The discovery and clinical implementation of immune checkpoint inhibitors has revolutionised the management of metastatic bladder cancer, including agents that inhibit the checkpoint target programmed death ligand-1 (PD-L1), which is expressed on the surface of tumour cells.[61] The combination of immune checkpoint inhibition with bladder-sparing TMT could be a promising therapeutic approach, because of the potential synergy between radiation and immunotherapy.[62] However, only a subset of bladder cancers respond to immune checkpoint inhibitors and identifying predictive biomarkers represents a crucial unmet need. In a cohort of 72 patients with muscle-invasive bladder cancer treated with TMT, expression of PD-L1 in bladder tumours was associated with low proportions of patients achieving a complete response and high numbers of patients with locoregional failure.[25] However, the immunohistochemical reagents, methods, and cutoff values used to quantify PD-L1 expression were different than methods that have been used to quantify PD-L1 expression in non-TMT cohorts, and the role of PD-L1 expression as a prognostic or predictive biomarker in the context of chemoradiation requires further study.

Genomic instability caused by loss of DNA repair function can activate an anti-tumour immune response through the production of tumour-specific neoantigens or the activation of the innate immune system. Tumour DNA repair deficiency has emerged as one of the most promising genomic biomarkers of sensitivity to immune checkpoint inhibitors.[63] The most well-characterised link between tumour DNA repair deficiency and response to immune checkpoint inhibitors exists for tumours that have a defect in the mismatch repair pathway,[64] which is frequently associated with the microsatellite instability phenotype. Indeed, pembrolizumab (an anti-PD-L1 agent) was recently approved to be used in patients with mismatch repair-deficient tumours or microsatellite instability-high tumours. The association between alterations in DNA repair pathways beyond mismatch repair and sensitivity to immune checkpoint inhibition is less well established; however, several recent reports show an association between alterations in DNA damage response genes and improved response to immune checkpoint inhibitors (alone or in combination with conventional chemotherapy) in patients with metastatic bladder cancer.[65–67]

Whole-exome sequencing analyses have shown a high somatic mutation frequency in muscle-invasive bladder cancer (median of 5·8 mutations per megabase),

a frequency similar to that seen in lung cancer and melanoma.[28,43] Mutation burden, quantified as the number of single-nucleotide variants per megabase, has been associated with response to anti-PD1 and PD-L1 agents.[68] Furthermore, distinct mutational signatures in bladder tumours have been associated with events such as apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like (APOBEC)-mediated mutagenesis and somatic *ERCC2* mutations,[69] and the activity of these mutational signatures has been associated with clinical outcomes (good or poor depending on the clinical context). Combining genomic and clinical data from TMT cohorts will provide important insights regarding the association of specific mutational processes with response to TMT and their potential for synergistic response with the addition of immunotherapy.

In a recent study using microarray-based whole transcriptome gene-expression profiling of TURBT specimens from 136 patients with muscle-invasive bladder cancer treated with bladder preservation therapy, we found that decreased gene-expression signatures of T-cell activation and interferon gamma signalling were associated with worse disease-specific survival.[27] In a comparison cohort of patients with muscle-invasive bladder cancer treated with neoadjuvant chemotherapy and radical cystectomy, no differences were found in disease-specific survival based on immune signature. Conversely, increased activity of a gene-expression signature of stromal infiltration was associated with worse disease-specific survival in the cystectomy cohort, but not in the bladder preservation cohort.[27] These findings suggest that immune and stromal expression signatures might be used as predictive biomarkers for bladder preservation therapy and could also have implications for predicting a benefit for the addition of immunotherapy to TMT, because of known interactions between immune cells and stromal infiltration and response to immune checkpoint blockade in metastatic bladder cancer.[70,71] Thus, these biomarkers and other genomic biomarkers could help direct therapeutic decision making and inform rational approaches to combine immunotherapy with bladder preservation therapy.

Liquid biopsies

The molecular biomarkers discussed so far are based on the analysis of bladder tumour biopsies, primarily obtained in the pre-treatment setting through cystoscopy and TURBT. By contrast, liquid biopsy biomarkers based on the analysis of blood and urine samples are non-invasive approaches that can be performed serially, thus enabling the monitoring of therapeutic responses and the early detection of relapse after treatment (figure 1).[72,73] Blood-based liquid biopsies including circulating tumour cells and circulating tumour DNA (ctDNA) have been explored for their potential utility as prognostic biomarkers in muscle-invasive bladder cancer and as tools for disease monitoring.[73,74] For example, in a study of 100 patients with muscle-invasive bladder cancer treated with radical cystectomy, circulating tumour cells were detected in 23% of patients pre-operatively and their presence was an independent predictor of disease recurrence, cancer-specific mortality, and overall mortality.[74] In the setting of postoperative disease monitoring after radical cystectomy, ctDNA measurements of bladder tumour-specific mutations in plasma were found to enable the early detection of metastatic relapse with a lead time of 101 days before radiographic detection.[73] Although such liquid biopsy biomarkers have not yet been tested extensively in the setting of bladder preservation therapy, they could be used as complementary approaches to tumour-based molecular biomarkers, and therefore should be investigated further.

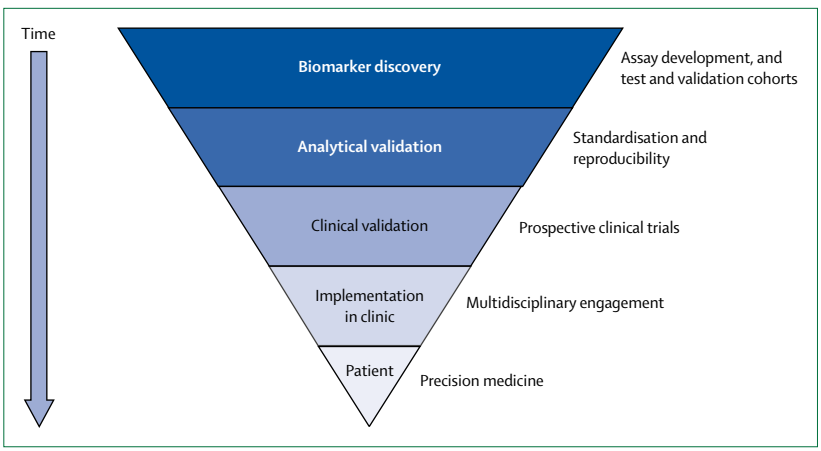

Figure 4: **Clinical implementation of biomarkers**
The pool of candidate biomarkers shrinks during each stage of development. Ultimately, only around 0·1% of candidate biomarkers are successfully translated into routine clinical care.

Molecular imaging

Recent advances in imaging technologies have given rise to powerful non-invasive functional imaging techniques such as diffusion-weighted imaging (DWI)-MRI, which detects the degree of diffusivity of water molecules in tissues and hence provides information about tissue pathophysiology. Molecular imaging techniques such as DWI-MRI could be used as non-invasive prognostic biomarkers in the setting of bladder preservation therapy for muscle-invasive bladder cancer. For example, in a cohort of 23 patients with muscle-invasive bladder cancer undergoing bladder-sparing therapy based on chemoradiation, a low pre-treatment apparent diffusion co-efficient value as measured by DWI was associated with a high pathological complete response rate after chemoradiation.[75] Additionally, DWI can be used for the non-invasive monitoring of therapeutic response after chemoradiation and is superior for assessing response compared with standard MRI techniques, including T2-weighted imaging and dynamic contrast-enhanced T1-weighted imaging.[76] A standardised approach to imaging and reporting multiparametric MRI for bladder

cancer has recently been developed (Vesical Imaging-Reporting and Data System), which could be useful to determine response to bladder-sparing therapy.[77] However, further improvements in sensitivity are necessary for the detection of small lesions and microscopic recurrences, and thus these imaging technologies will need to be further evaluated in the context of standard cystoscopic biopsies and tissue-based molecular biomarkers.

Towards clinical implementation

Although numerous promising molecular biomarkers have been described for muscle-invasive bladder cancer (table), the path to clinical implementation has been difficult, as evidenced by an estimated 0·1% rate of successful clinical translation of biomarkers (figure 4).[78] An important process is analytical validation of the biomarker, which assesses the accuracy and reliability of the test measurement in a patient specimen.[79] Analytical validation includes the standardisation of the assay platform and a clear demonstration of the robustness and reproducibility of measurements. In the USA, biomarker assays that will ultimately be used in the clinic should be done in diagnostic laboratories certified for Clinical Laboratory Improvement Amendments, with established analytical performance specifications and quality control procedures necessary for eligibility. Moreover, practical issues need to be considered, including availability and cost of the assay, and turnaround time, which is an important consideration when a biomarker is intended to guide time-sensitive clinical decisions.

The implementation of any biomarker in routine clinical practice will also require its validation in prospectively planned clinical trials. In this respect, none of the biomarkers discussed in this Review have yet been formally validated in a prospective clinical trial. Such studies should include in their design the a-priori specification of a hypothesis regarding the prognostic or predictive value of the biomarker, and the blinded measurement of the biomarker regarding clinical outcomes.[80] Sargent and colleagues[80] proposed two different clinical trial designs for the formal validation of predictive biomarkers in cancer treatment trials. The first is a "marker by treatment interaction design",[80] in which subjects are stratified by biomarker in the context of a phase 3 clinical trial and a formal test of interaction is used to detect differential treatment effects in the biomarker groups. The second is a "marker-based strategy design",[80] in which subjects are randomly assigned to either biomarker-guided treatment selection or standard treatment selection independent of biomarker status.

The incorporation of biomarkers into clinical trials will require coordination between investigators and molecular diagnostics companies, both in the context of smaller institutional studies and larger national and international cooperative group studies. These efforts will probably not be easy—but will be valuable regardless of their findings. For example, Stadler and colleagues[81] reported the results of a phase 3 trial that selected patients with p53-positive pT1/T2 N0 M0 bladder cancers for randomisation to adjuvant methotrexate, vinblastine, doxorubicin, and cisplatin (MVAC) chemotherapy versus observation. Although the findings from this trial did not show the prognostic or predictive value of p53, they highlighted the feasibility of doing multi-institutional bladder cancer trials that base treatment decisions on standardised, centrally-performed molecular biomarker assays. One example of an ongoing prospective biomarker trial for muscle-invasive bladder cancer is the Co-expression Extrapolation (COXEN)/SWOG study (NCT02177695), in which patients are randomly assigned to receive either dose-dense MVAC or gemcitabine and cisplatin as neoadjuvant chemotherapy before radical cystectomy and uses a COXEN gene expression score.

Several planned or ongoing clinical trials of bladder preservation therapy for muscle-invasive bladder cancer have incorporated molecular biomarkers and genomic analyses into their trial design, with the goal of defining biomarkers associated with treatment response and outcomes. The SWOG S1806 trial will randomly assign patients with muscle-invasive bladder cancer to chemoradiation with or without the anti-PD-L1 checkpoint inhibitor atezolizumab, and will include transcriptional profiling and comprehensive genomic analysis of all samples. The results from this trial will not only evaluate the potential role for adding immunotherapy to chemoradiation as part of bladder preservation therapy but will also provide prospective data for the role of molecular subtype, mutational burden, and other molecular features (including MRE11 protein expression) as potential biomarkers of treatment response. Additionally, two biomarker-driven phase 2 trials are investigating a bladder preservation strategy involving chemotherapy alone (NCT02710734, NCT03609216), in which patients with muscle-invasive bladder cancer whose tumours exhibit a mutation in pre-defined DNA repair genes. Those patients who achieve a complete clinical response to neoadjuvant chemotherapy will avoid immediate radical cystectomy or radiotherapy and will instead undergo surveillance with cystoscopies. These studies represent promising initial steps to personalise treatment for bladder cancer therapy through the use of biomarkers, in the context of bladder-sparing TMT and beyond. Hopefully, advances in biomarker development and validation, and the incorporation of biomarkers into trials will lead to changes in how bladder cancer is managed in the future.

Conclusions

Recent advances in our molecular understanding of bladder cancer have led to the discovery of molecular biomarkers associated with patient outcomes after bladder preservation therapy. Although prognostic biomarkers are important, predictive biomarkers that reflect the intrinsic biology of the tumour and can be used to

predict response to specific therapies are of particular interest. Such predictive biomarkers can provide rational guidance regarding the most appropriate therapy for each individual patient and can assist in the decision-making process when choosing between radical cystectomy and bladder preservation therapy for the treatment of patients with muscle-invasive bladder cancer. Biomarkers could also potentially be used to design optimal therapeutic regimens for individual patients, such as the addition of immunotherapy to chemoradiation in biomarker-selected subsets of patients with muscle-invasive bladder cancer. These biomarkers require rigorous standardisation of analytical methods and prospective validation in clinical trials before they can be routinely implemented in the clinic. However, with the rapid emergence of promising new molecular biomarkers and the development of clinical trials to validate them, we will hopefully soon have the tools necessary to practise precision medicine in the management of bladder cancer.

Contributors

DTM, KWM, FYF, and JAE did the literature search. DTM, KWM, and JAE created the figures. All authors wrote and edited the manuscript and approved the final version of the manuscript.

Declaration of interests

KWM reports personal fees from Pfizer and EMD Serono outside the submitted work. FYF reports personal fees from Janssen, Medivation/Astellas, Sanofi, EMD Serono, Bayer, and Ferring, and co-founding of PFS Genomics, all outside the submitted work. JAE reports personal fees from Janssen, Genentech, EMD Serono, Bayer Healthcare, Blue Earth Diagnostics, and Taris Biomedical, all outside the submitted work. DTM and WUS declare no competing interests.

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