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Review

Invited Review: Biomarkers in Bladder Cancer

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Abstract

Cancer biomarkers provide an opportunity to diagnose tumours earlier and with greater accuracy. They can also identify those patients most at risk of disease recurrence and predict which tumours will respond to different therapeutic approaches. Such biomarkers will be especially useful in the diagnosis and management of bladder cancer. At present bladder tumours are diagnosed and followed up using a combination of cystoscopic examination, cytology and histology. These are not only expensive, but also highly subjective investigations and reveal little about the underlying molecular characteristics of the tumour. In recent years numerous diagnostic and prognostic biomarkers of bladder cancer have been identified. Two separate approaches to biomarker discovery have been employed. The first is hypothesis-driven and focuses on proteins involved in molecular pathways known to be implicated in tumourigenesis. An alternative approach has been to study the global expression of genes (so-called 'genomics') looking for characteristic signatures associated with disease outcomes. In this review we summarise the current state of biomarker development in this field, and examine why so few have made the successful transition into the clinic. Finally, we introduce a novel approach to biomarker development utilising components of the DNA replication licensing machinery.

Introduction

Bladder cancer is the sixth most common malignancy in the UK with over 10,000 new cases diagnosed annually and is responsible for the deaths of 4,800 people each year. Like all cancers, the prognosis and clinical management of bladder cancer is strongly dependent upon the stage at which it is first diagnosed. The majority (75%) of patients present with superficial (pTa and pT1) tumours and have a 5-year survival rate of between 88-98%. In contrast the 5-year survival rate for patients presenting with muscle invasive disease (pT2-pT4) is 46-63%, and falls to only 15% in those with locally advanced and metastatic disease*. In addition to pathological stage, histological features such as tumour grade are also independent predictors of disease outcome¹. The accurate histological assessment of non-invasive tumours, commonly divided into 'papillary' and 'non-papillary' subtypes, is especially important. Non-papillary tumours, more commonly referred to as 'carcinoma in situ' (CIS), are invariably aggressive high grade lesions which frequently progress to invasive cancer. In contrast superficial papillary tumours are typically lower grade lesions which behave less aggressively.

Despite the excellent prognosis associated with superficial tumours, almost 50% of patients will experience recurrence of their disease within 4 years of their initial diagnosis, and 11% will progress to muscle invasive disease². Therefore, the successful management of bladder cancer requires not only early diagnosis, careful histological assessment and accurate staging, but also frequent and long-term surveillance.

The current gold standard for the diagnosis and surveillance of bladder cancer remains direct visualisation of tumours by conventional white light cystoscopy. In addition, urine-based

* National Cancer Institute (NCI), SEER database: www.seer.cancer.gov/publications/survival/surv_bladder.pdf

cytology is used to screen symptomatic and high-risk patients and is a useful adjunct in long-term surveillance protocols. The histological assessment of biopsy material taken during cystoscopic examination provides important information about tumour type, size, grade, degree of muscle invasion, and the presence or absence of lymphovascular invasion.

By combining histological, radiological and clinical information, tumours can be staged using the TNM system. This established staging system is an indicator of tumour burden and the extent of spread and is widely used as a predictor of disease outcome and to guide treatment (e.g. local vs systemic; conservative vs aggressive). In bladder cancer it is possible to further refine estimates of disease outcome through the development of prognostic indices, which include clinical variables such as stage, tumour number, grade, presence/absence of CIS and history of previous recurrence²⁻⁴.

Whilst the widespread use of cystoscopy and urine cytology has contributed to a significant decline in the mortality associated with bladder cancer, both suffer from significant drawbacks as diagnostic and screening tests. Cystoscopy is technically demanding and invasive procedure which exposes the (often elderly) patient to significant iatrogenic risks, including infections, bleeding and perforation as well as complications arising from anaesthesia. Whilst cystoscopic examination identifies nearly all papillary and sessile lesions, small areas of CIS can be easily missed. Furthermore, the histological assessment of tumour grade in biopsies of superficial disease is highly subjective⁵⁻⁷. This is reflected in recent changes to the WHO (2004) classification of non-invasive disease which replaces the original division of tumours into grades 1, 2 and 3 (WHO 1973) with two, low and high grade categories⁸⁻¹¹. Whilst this re-classification is likely to improve patient outcome, the elimination of the commonly used grade 2 category has proven contentious and has yet to be

fully validated in prospective studies¹². The difficulties presented by histological grading are further compounded by tumour heterogeneity and multicentricity.

Despite a high specificity (78-100%), urine cytology lacks robust sensitivity (12.2-84.6%), especially for low grade tumours, and is subject to considerable inter-observer variability¹³. Finally, cystoscopy and cytology are expensive techniques, especially when used in long-term surveillance programmes. In the US it has been estimated that the cost per patient of bladder cancer from diagnosis to death is the highest of all cancers, ranging between \$96,000 and \$187,000¹⁴.

Cancer Biomarkers

The high cost and limitations of the current diagnostic and screening tests have led many to seek alternative markers of bladder cancer. Ideally these cancer 'biomarkers' would allow earlier diagnosis, show higher sensitivity and specificity, be more reliable and easier to perform and reduce overall costs.

A search of the current literature returns a bewildering array of reports relating to biomarker studies in virtually all areas of medicine, and bladder cancer is no exception. Moreover, the terminology used to describe these biomarkers and the context in which they are used can be confusing and often misleading. A widely accepted and comprehensive definition describes a biomarker as "*a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacological responses to a therapeutic intervention*"¹⁵. Within this broad definition, biomarkers specific to cancer are typically classified as either 'diagnostic', 'prognostic' or 'predictive'. Diagnostic cancer biomarkers are those markers which can identify the presence (or absence) of a neoplastic

process in a given setting, ideally before there are clinical manifestations. Such biomarkers can also be used as a screening test and to monitor the response to therapy and are already used routinely in the diagnosis and surveillance of other genitourinary malignancies such as prostate (e.g. PSA) and testicular cancers (e.g. β hCG). Prognostic biomarkers are those markers which have an association with clinical outcome, typically overall and recurrence-free survival. Such markers can be exploited as decision-making aids to help identify which patients would benefit most from chemotherapy and radiotherapy, and how aggressive this treatment should be ¹⁶. In contrast, predictive biomarkers can be used to make more specific choices between treatment options. For example, oestrogen receptor (ER), progesterone receptor (PR) and Her2 receptor testing is now routinely used to select breast cancer patients for treatment with tamoxifen and trastuzumab[†] ¹⁷, whereas genetic screening of patients with colorectal cancer for K-ras mutations is used to identify individuals who will not respond to cetuximab ¹⁸.

In contrast to tumour grading and staging, which fail to take account of the molecular diversity of cancer, prognostic and predictive biomarkers offer the ability to draw specific conclusions about individual patients and their tumours, providing so-called 'personalised medicine'. The ability to use such markers to tailor treatment to the individual is both cost effective and avoids unnecessarily exposing patients to the potentially harmful side effects of such treatment. However, despite years of research and almost 100,000 published reports, the number of cancer-specific biomarkers in clinical use remains surprisingly small ¹⁹ (Figure 1). Whilst initial reports are often promising, subsequent studies on the same or related markers

[†] Trastuzumab for the adjuvant treatment of early-stage HER2-positive breast cancer. National Institutes for Clinical Excellence (NICE), August 2006. www.nice.org.uk/nicemedia/pdf/TA107guidance.pdf

frequently yield contradictory findings. These constraints are discussed below in relation to biomarkers that have undergone clinical evaluation.

Diagnostic Biomarkers

The current paradox in biomarker development is very familiar to specialists involved in the diagnosis and treatment of bladder cancer. However, a handful of diagnostic and screening biomarkers have gained approval from the US Food and Drug Administration (FDA) and are now commercially available (Table 1).

Bladder Tumour Antigen test

The Bladder Tumour Antigen (BTA) test measures levels of complement factor H-related protein in the urine. This protein is similar to factor H and high levels are secreted by tumour cells into the urine ²⁰. There are two forms of the test: BTA-Stat, a point-of-care immunoassay, and BTA-Trak, a laboratory-based enzyme-linked immunosorbent assay (ELISA). Whilst both tests outperform cytology on sensitivity (24-89% and 51-79% respectively), the reported specificity falls below that of cytology, and false positive results are common in benign inflammatory and infectious conditions ^{21,22}.

Nuclear matrix protein 22

Nuclear matrix protein 22 (NMP22) is a regulator of mitosis and its expression is significantly increased in malignant urothelium. NMP22 is released into urine following apoptosis and can be measured by a quantitative immunoassay (NMP22 Test) or a qualitative point-of-care test (NMP22 BladderChek) using monoclonal antibodies. Numerous studies have demonstrated that NMP22 has a superior sensitivity (46-88% and 40-85% respectively) to urine cytology ¹³. Furthermore, in a large prospective study of 668 patients with a history

of bladder cancer, the combination of NMP22 and cystoscopy identified 99% of all tumours compared to only 91.3% with cystoscopy alone²³. However, the specificity varies widely depending upon the cut-points used and typically falls below that of urine cytology^{13, 24}. Furthermore, like BTA Trak, the false positive rate is significantly raised in patients with benign inflammatory conditions^{25, 26}.

ImmunoCyt

ImmunoCyt is an immunofluorescence-based test in which a cocktail of three antibodies are used to detect two mucin-like proteins and a high molecular weight form of carcinoembryonic antigen²⁷. These molecules are present on the surface of exfoliated malignant urothelial cell. The overall reported sensitivity and specificity rates of this test are good (63-85% and 62-79% respectively), but specificity remains below that offered by conventional cytology^{13, 24}. Furthermore, the test requires the use of a fluorescence microscope by a trained operator and there are reports of interobserver variability²⁸. As a result, this test is currently recommended only as an adjunct to cytological examination.

UroVysion

UroVysion is also a fluorescence-based assay, but uses fluorescence in situ hybridisation (FISH) to identify common alterations in chromosomal copy number and loss of the 9p21 locus²⁹. A number of studies have shown that this test has high sensitivity (69-96%) and a specificity (65-96%) that almost matches urine cytology^{13, 24}. Furthermore, since this test identifies neoplastic genetic alterations, it is largely unaffected by benign inflammatory conditions, and recent studies suggest that previously reported false positive tests may in fact represent early sub-clinical neoplastic changes^{30, 31}. However, UroVysion is an expensive test requiring specialist equipment and diagnostic expertise.

Owing to their inferior specificity and lack of robust prospective testing, none of these tests is currently recommended by international or European guideline panels^{32, 33} (see also www.uroweb.org). However, improvements in both specificity and sensitivity have been reported using combinations of these biomarkers, and many advocate their use in extending the time between surveillance cystoscopy, especially in low grade non-invasive disease and in cases where cytology was equivocal³⁴⁻³⁷.

Prognostic Biomarkers

Whilst there has been considerable focus on the development of diagnostic biomarkers, clinicians and their patients are becoming increasingly eager to exploit any new test that can provide more accurate prognostic information or better direct the use of both conventional and emerging therapeutic agents. The search for such biomarkers has begun in earnest. Initial studies have focused on the observation that the malignant transformation of urothelial cells appears to arise through alterations in key molecular pathways and that distinct pathways may be involved in the development of invasive and non-invasive forms of the disease³⁸⁻⁴⁰ (Figure 2).

FGFR/MAPK pathway

Pathways involved in cell growth signalling are frequently dysregulated in low grade, non-invasive tumours. Over 70% of these tumours harbour activating mutations in the fibroblast growth factor receptor 3 (FGFR3) gene compared to only 10-20% of invasive tumours⁴¹⁻⁴⁴. The mutations appear to result in overexpression of FGFR3 and constitutive activation of the Ras-MAPK pathway increasing signal transduction to transcription factors in the nucleus. Interestingly, mutations in FGFR3 and Ras appear to be mutually exclusive but together

account for well over 80% of all low grade tumours indicating that activation of the Ras-MAPK pathway may be an obligate event in most low grade tumours⁴⁵. Furthermore, overexpression of FGFR3 has also been reported in a significant proportion (42%) of tumours with no identifiable mutations, suggesting that increased FGFR3 signalling can also contribute to malignant transformation in early bladder cancer⁴⁶. Although a number of studies have shown promising results, the prognostic value of FGFR3 mutations and protein expression levels remains controversial⁴⁷⁻⁵⁰.

p53 and cell cycle regulation

In contrast to superficial tumours, CIS and invasive tumours show frequent alterations in the TP53 tumour suppressor gene⁵¹. Loss of heterozygosity (LOH) studies have demonstrated that chromosome 17p, the location of TP53, is lost in 60% of invasive cancers^{52, 53}. Such loss is a rare event in non-invasive tumours. Alterations in related regulatory molecules such as the cyclin-dependent kinase (CDK) inhibitors p21 and p16 have also been linked to increased disease recurrence and progression to invasive disease^{54, 55}. Initial studies of mutant p53 expression demonstrated a strong association with disease grade and stage, as well as a significantly higher risk of recurrence and decreased survival⁵⁶. However, a recent meta-analysis of 168 published reports found such associations to be weak and inconsistent⁵⁷. This view was supported by an international panel which concluded that whilst p53 showed promise as a prognostic biomarker, the data remain inconclusive⁵⁸. Studies examining alterations in the expression of p16 and p21 also show contradictory findings^{54, 59-63}, although their prognostic significance did improve significantly when incorporated into a larger panel including p53⁶⁴⁻⁶⁶.

Cyclins D1 and E

Alterations in expression of cyclins D1 and E have also been observed in bladder cancer. Cyclin D1 and cyclin E are both important cell cycle regulators controlling G1 phase progression and the G1/S phase transition respectively. Their dysregulation has been linked to uncontrolled cell proliferation and establishment of autonomous cancer cell cycles. Unfortunately, the prognostic significance of cyclins D1 and E also appears uncertain. Although early studies linked cyclin D1 expression to disease recurrence⁶⁷, this observation is not widely supported and a number of studies have demonstrated that there is no association between cyclin D1 expression and prognosis^{62, 68, 69}. Other studies have shown that low cyclin E expression is associated with increased disease recurrence, progression and reduced overall survival⁶⁹⁻⁷¹. However, these findings are also widely disputed and appear to be lost on multivariate analysis^{70, 72}.

Ki67

Ki67 is a commonly used marker of cell proliferation and has been extensively studied in almost all cancers. Since Ki67 is only expressed by cells actively engaged in the cell cycle, positive immunostaining is considered a good correlate of biological 'aggressiveness'. Numerous studies have identified Ki67 as an independent prognostic marker of disease recurrence, progression and disease-specific survival⁷³⁻⁷⁵. Whilst variation in study design and assay interpretation has resulted in a number of contradictory reports, a large multicentre study has confirmed that Ki67 is independently associated with disease recurrence, overall survival and could be used to predict which patients might benefit from preoperative systemic chemotherapy⁷⁶.

Apoptosis

Dysregulation of the apoptotic signalling pathways plays an important role in tumourigenesis, allowing the continued survival of genetically damaged tumour cells. The prognostic significance of the anti-apoptotic gene Bcl-2 in bladder cancer has been studied by a number of groups. Some have shown that increased expression of Bcl-2 is associated with aggressive disease and poor outcome⁷⁷⁻⁷⁹, whereas others have questioned this association^{75, 80}. A number of recent studies have highlighted the potential importance of survivin, which is known to regulate mitotic progression and enhance angiogenesis⁸¹⁻⁸³. A number of studies have demonstrated a strong association between overexpression of survivin and disease recurrence and progression^{79, 84}, a finding recently confirmed in a large multicentre study⁸⁵. However, the association was relatively small and the authors reported that there was no statistical or clinical benefit to be gained from adding survivin to standard histopathological criteria for the prediction of outcome.

Dissection of the molecular pathways involved in the pathogenesis of bladder cancer has provided a rich hypothesis-driven source of potential prognostic biomarkers. These individual markers are quantifiable and show some association with disease behaviour and outcome. However, few of these markers have proven themselves in the clinical arena and none has been adopted into routine practice. This failure arises in part due to the varied approach to study design (retrospective vs prospective), patient selection and statistical analysis employed by different research groups. In particular, many of the studies described above used immunohistochemical-based assays to quantify protein expression. Whilst this technique has revolutionised diagnostic histopathology in recent years, it is a technique which can be difficult to standardize. Differences in a number of critical variables such as the type of antibodies used, antigen retrieval methods, reagents, pH, temperature, and the quality of the

tissue are all likely to contribute to the varied and often contradictory findings reported in prognostic biomarker studies.

In an attempt to improve and standardise current prognostic biomarker development, McShane et al. published a set of reporting criteria under the acronym 'REMARK' (REporting recommendations for tumour MARKer prognostic studies)⁸⁶. The report lists 20 recommendations outlining specific requirements for study design, patient and specimen characteristics, assay design and statistical methodology. Many funding organisations and journals have adopted these recommendations and now include them in their reporting criteria. Methodological standardization in immunohistochemistry is also being addressed through the development of national quality assurance schemes such as the UK National External Quality Assessment Service for Immunocytochemistry (NEQAS-ICC, see www.ukneqasicc.ucl.ac.uk) which aim to improve the quality and reproducibility of this technique through education and dissemination of best practice.

In addition to the problems addressed by the REMARK guidelines and national quality assessment schemes, the inability to identify robust prognostic biomarkers also reflects a naive approach to tumourigenesis. Whilst a number of important molecular pathways have been identified in bladder cancer, the mechanisms underlying the malignant transformation and metastatic potential of a tumour cell will in reality depend upon complex and often redundant interconnecting networks of regulatory proteins. The diagnostic waters are further muddied by the diverse genetic background of individual patients, and in such circumstances the identification of lone markers of such complex disease behaviour appears overly optimistic. Indeed, the recent publication of the first cancer genome has demonstrated that a tumour cell might typically carry over 30,000 genetic mutations⁸⁷.

Genomic Profiling

Fortunately, the emerging field of high-throughput transcript profiling using genomic and proteomic technologies has provided researchers with the means to study the global expression of genes and their related proteins within individual tumours. This approach does not require an initial hypothesis and so is not restricted to the study of specific molecular pathways. Instead characteristic molecular signatures emerge from the 'transcriptome' and are linked to measures of disease outcome.

Whilst this technology has been most comprehensively applied to the study of breast cancer^{88, 89}, a growing number of genomic studies have been reported in bladder cancer. These studies have identified unique gene expression signatures associated with disease stage⁹⁰⁻⁹⁴, progression^{95, 96}, recurrence⁹⁰, nodal metastases⁹⁷ and response to chemotherapy^{98, 99}. Notably Dyrskjot and co-workers demonstrated that tumours histologically defined as superficial but which expressed an invasive gene signature displayed a significantly higher probability of subsequent disease progression and poor outcome⁹⁰. These findings demonstrate the potentially superior prognostic and predictive power that gene expression signatures may have over conventional diagnostic techniques. It is also encouraging that, whilst these technologies identify genes in an unbiased manner, the molecular signatures appear enriched for genes involved in pathways already known to be important in carcinogenesis. One of the most prominent of these relates to cell proliferation and the cell cycle and is observed in many different cancer-specific gene signatures.

However, whilst gene expression profiling has undoubtedly provided new insights into the pathogenesis of bladder cancer and the search for biomarkers, the reliability and

reproducibility of gene expression profiling has been widely questioned¹⁰⁰⁻¹⁰². For example, there is little or no correlation between the gene signatures reported in different studies^{91, 95, 103} and many fail to provide a significantly improved prognostic classification when compared to conventional clinicopathological criteria. There are several reasons for the apparent failure of gene expression profiling. Many of the studies performed to date have analysed very small patient cohorts, typically drawn from retrospective studies and/or archival tissue banks, and lack sufficient independent validation. Validation across different analysis platforms, methodologies, laboratories and patient groups is essential, as is testing in prospective studies, if this technology is to be adopted into routine clinical use. Expression profiling of resected tumours and biopsy material also suffers from sampling error as RNA/DNA/protein are typically extracted unselectively. This is especially important when considering proliferation signatures since tumours commonly comprise a heterogeneous population of neoplastic cells, many of which will not be actively cycling¹⁰⁴. The presence of contaminating benign neoplastic cells, lymphoid follicles, inflammatory cells and reactive stroma further distort analysis by adding potentially large numbers of cells with complex cell cycle kinetics. Finally, the cost of genomic-based tests are likely to be considerable. For example, Oncotype DX (Genomic Health) and MammaPrint (Agendia), have recently been introduced as tests which may improve risk assessment in early stage breast cancer, but each costs in excess of \$3,500 per test.

DNA Replication Licensing Factors as Diagnostic and Prognostic Biomarkers

Analysis of upstream signalling pathways controlling cell proliferation, differentiation, apoptosis and DNA damage response by conventional hypothesis-driven molecular approaches and genome wide analyses remain unproven as routine tools for clinicopathological assessment of malignant tumours. An alternative approach is to focus on

key regulators of the cell cycle machinery, which act as an integration point for information transduced through upstream signalling pathways. We and others have shown that expression of Mcm2-7 (MCM) proteins, constituents of the DNA replicative helicase and essential for DNA replication initiation¹⁰⁵, can be used to determine the cell cycle, state in human tissues¹⁰⁶. MCMs are strongly expressed by cells during all stages of the proliferative cell cycle but are absent in quiescent, terminally differentiated and senescent out-of-cycle states, and as such represent new biomarkers of growth^{107, 108}.

Mcm5

Since neoplastic cells are characterized by uncontrolled proliferation, MCM expression has been extensively investigated as a diagnostic marker in a broad range of neoplastic conditions including bladder cancer¹⁰⁹⁻¹¹⁴. Immunohistochemical staining of normal urothelium with antibodies directed against Mcm5 shows positive staining restricted to the basal stem-transit proliferative compartment¹¹⁵. In contrast, both non-invasive and invasive urothelial carcinomas show Mcm5 staining in all layers of the urothelium including superficial epithelial cells, reflecting the arrested maturation of these tumour cell populations and their inability to exit the proliferative cycle. This results in the exfoliation of Mcm5-expressing tumour cells into the urine. Mcm5 protein levels can be accurately measured in urine sediments using a simple automated immunofluorometric assay. Application of this assay in a blinded clinical trial has demonstrated that elevated Mcm5 levels in urine sediments is highly predictive of bladder cancer and superior to urine cytology¹¹⁶. Notably, unlike many of the commercially available diagnostic biomarkers discussed earlier, the specificity of the Mcm5 test is not affected by benign proliferative or inflammatory conditions, including bacillus Calmette-Guerin (BCG) induced granulomatous cystitis. Moreover, this study also

demonstrated that the Mcm5 urine test is able to detect early (organ confined) prostate cancer.

Cell cycle algorithm

The utility of MCM proteins as cancer biomarkers is further enhanced by combining them with other key cell cycle regulators. Geminin, a repressor of MCM loading at origins of DNA replication, is present only in S, G2 and M phases, and can therefore act as a marker for S-G2-M phase progression¹¹⁷. Similarly, protein levels of the mitotic kinases Polo-like kinase 1 (Plk1) and Aurora A are negligible in G1, increase during S phase and reach a peak during G2/M, with rapid degradation occurring at the end of mitosis and therefore also represent biomarkers of S-G2-M phase progression¹¹⁸. Phosphorylation of the Aurora kinase substrate Histone H3 on serine 10 (H3S10ph) is restricted to mitosis, enabling its use as a mitosis-specific marker^{118, 119}.

By combining this panel of G1/S and G2/M regulators into a multiparameter algorithm, it is possible to provide a detailed analysis of the cell cycle kinetics of complex tumour cell populations. For example, in a recent study we have demonstrated that breast tumours can be classified into one of three cell cycle phenotypes: (I) out-of-cycle tumours in which tumour cells lack expression of all markers; (II) slowly cycling tumours in which only Mcm2 is expressed, indicating that the majority of cells reside within a prolonged or arrested G1 phase; (III) rapidly cycling tumours in which the tumour cells express high levels of S-G2-M and M phase markers¹²⁰. Importantly, patients with actively cycling phenotype III tumours have a significantly worse prognosis than those displaying phenotypes I and II. Furthermore, this prognostic readout is superior to both morphological grading and Ki67 on multivariate analysis^{118, 120}. Since grading and Ki67 expression are important prognostic indicators in

bladder cancer, the application of the multiparameter algorithm to these tumours may provide significant improvements in prognostication. This could be particularly helpful in the assessment of superficial, pTa and pT1 tumours, where the distinction between different grades remains highly subjective and controversial. Whilst many pathologists and clinicians continue to favour the 1973 WHO classification, this approach assigns a significant proportion of lesions to the intermediate or grade 2 category. Interestingly, initial studies of a randomly selected series of grade 2 tumours has demonstrated that all three cell cycle phenotypes are represented, further highlighting the constraints of conventional histological grading (Figure 3). In contrast to Ki67, the multiparameter algorithm also offers the ability to determine where in the cell cycle a tumour predominantly resides. As a result this will enable clinicians to identify those patients most likely to benefit from S and M phase specific chemotherapeutic agents.

In contrast to genomic studies which typically require specialised laboratory facilities as well as fresh tissue, expression levels of cell cycle proteins can be accurately quantified using relatively simple and inexpensive immunohistochemical staining of routinely processed paraffin-embedded material. This not only allows cell cycle dynamics to be easily determined in individual patient samples but also allows tumour-specific biomarker expression to be distinguished from that present within the background stroma. Moreover, since this approach focuses solely on the kinetics of cell proliferation and not specific molecules or pathways, it should be applicable to many different cancers, including bladder cancer.

Future directions

The search for more sensitive and specific biomarkers of bladder cancer continues. Further progress might be achieved by combining existing biomarkers into larger, more powerful

panels, or through the discovery of new biomarkers that robustly outperform current diagnostic tests and clinical indicators. A number of new approaches to biomarker discovery are currently being exploited in bladder cancer. These include molecular techniques such as proteomics, analysis of DNA methylation status and cancer specific microRNAs ¹²¹. Proteomics in particular, is a rapidly developing field of research which offers the opportunity to obtain a real time assessment of global protein expression and has been used to identify tumour-specific proteins secreted into bodily fluids including urine. By comparing protein expression between patients with bladder cancer and healthy controls or proteins expressed by invasive and non-invasive tumours, it is hoped that tumour-specific protein profiles can be identified which have diagnostic and prognostic value. However, like genomics, proteomics is a complex and expensive technique and currently lacks the reproducibility required for routine diagnostic and prognostic testing ¹²².

Despite numerous published studies, the number and quality of biomarkers available for use in bladder cancer remains disappointingly low. Regardless of the approach taken to identify biomarkers, it is essential that future studies are subjected to rigorous testing which must include prospective multicentre trials. The biopharmaceutical industry has enthusiastically embraced the development of molecular diagnostics and their involvement will certainly help fund improved research and development in this field. However, the introduction of single-use biomarker tests whose application is restricted to specific cohorts of patients will invariably result in high unit costs in the short term. However, despite these commercial constraints the search for diagnostic biomarkers is likely to continue. Both individual patients and healthcare providers are set to benefit from a new era of 'personalised medicine' in which biomarkers will direct the use of increasingly targeted and potentially more cost effective therapeutic agents.

For Peer Review

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For Peer Review

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Figure Legends

Figure 1. Graph showing the increasing number of publications identified in a Medline search using 'biomarker' as the medical subject heading (red squares) and as a text word (red circles). In contrast the green triangles show a decline in the number of FDA-approved plasma-protein markers. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Cancer¹⁹, copyright (2005).

Figure 2. Diagrammatic representation of a bladder showing proposed pathways involved in tumourigenesis and tumour stage. Invasive and superficial tumours are characterised by distinct alterations in specific pathways. Invasive carcinoma typically arises via carcinoma in situ in which p53 mutations occur early. These tumours display genetic instability and accumulate further genetic alterations, including Rb and p16 inactivation and deletion of chromosomal segment 8p. In contrast, superficial tumours classically show constitutive activation of the Ras-MAPK pathway, most commonly through activating mutations of FGFR3. The transition from low to high grade tumours is thought to involve a number of additional events including overexpression of cyclin D1, loss of chromosomal segment 11p and inactivation of p16. A proportion of superficial tumours will develop into invasive tumours following alterations in p53, p21 and/or loss of 8p.

Figure 3. Photomicrographs of three different grade 2 bladder cancers (WHO 1973 classification) each immunostained with four cell cycle biomarkers: Mcm2, geminin, Plk1 and H3S10ph. The bladder tumours (A-C) display cell cycle phenotypes I-III respectively. (A) Phenotype I is an out-of-cycle tumour in which all biomarkers show low expression. (B) Phenotype II is a G1 delayed/arrested tumour in which high Mcm2 labelling (pan-cell cycle

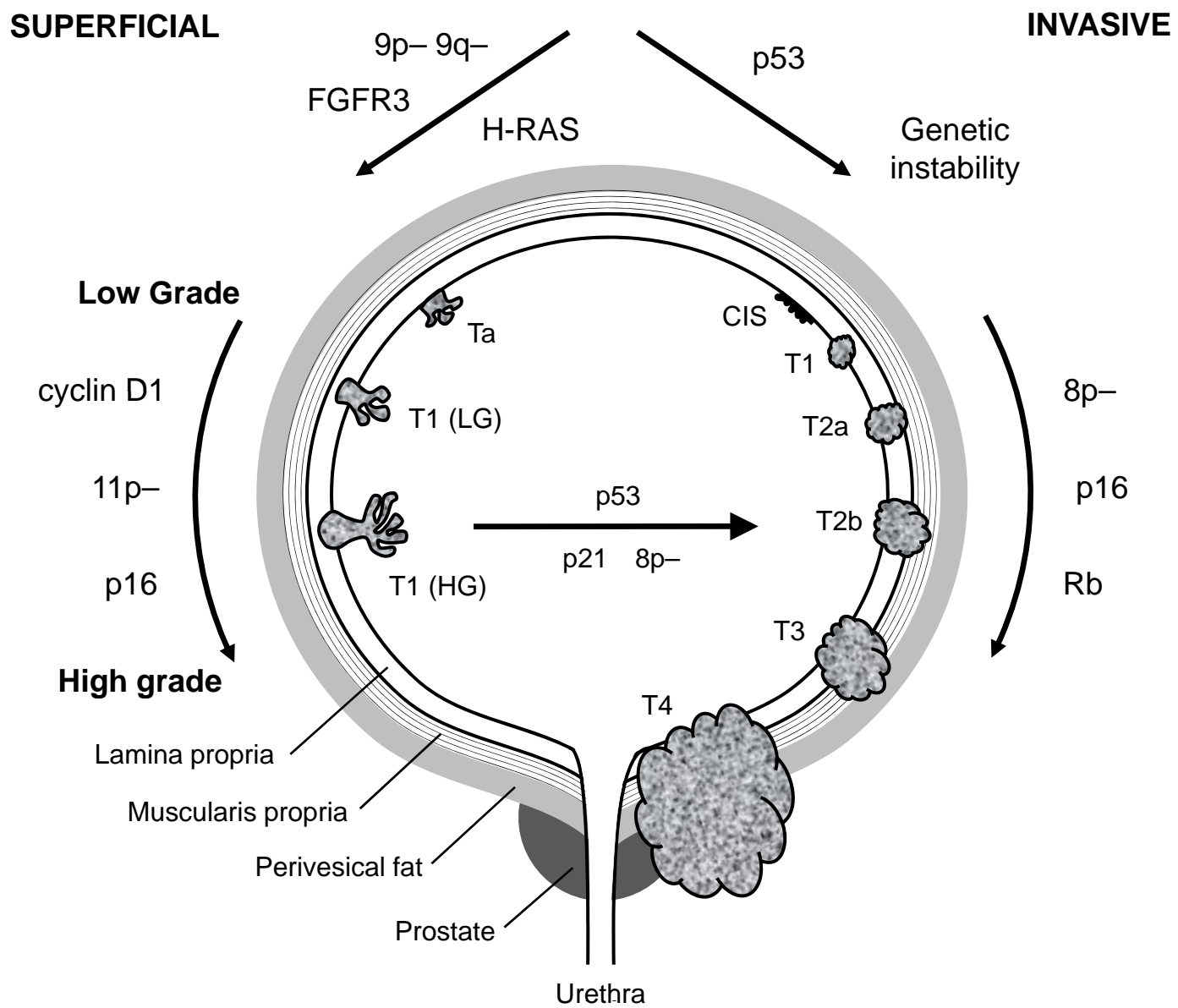
marker) indicates that the majority of cell are engaged in the cell division cycle. However low expression of geminin and Plk1 (S-G2-M phase markers) and H3S10ph (M phase marker) indicates failure of cell cycle progression. (C) Phenotype III is an actively cycling tumour indicated by high expression levels of Mcm2, geminin, Plk1 and increased H3S10 phosphorylation.

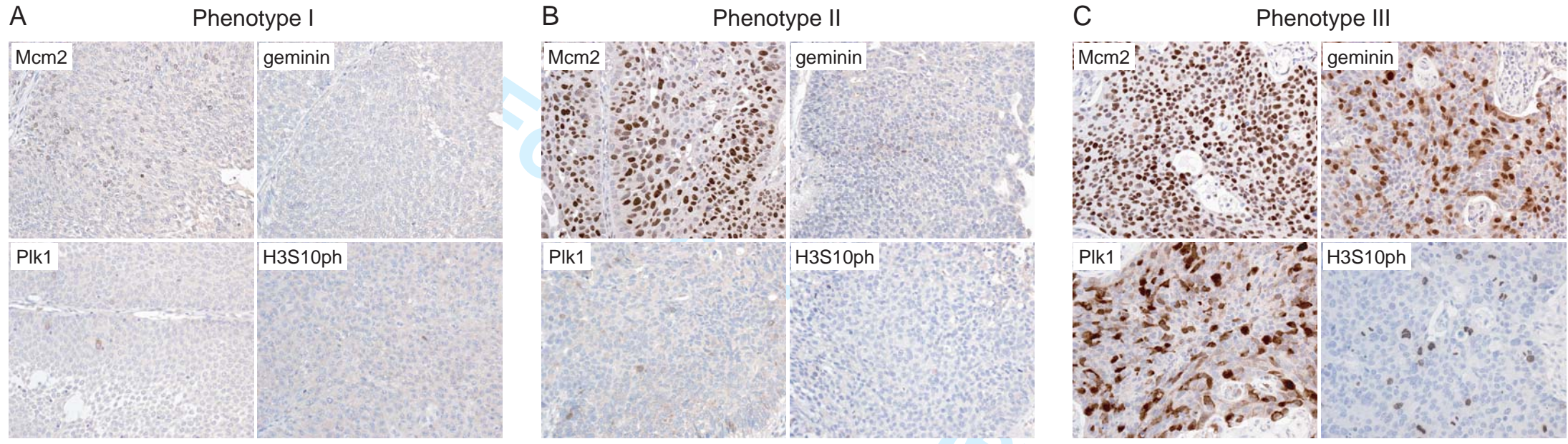
For Peer Review

Figure 1



Figure 2





Diagnostic Test	Specificity [†]	Sensitivity [†]	Advantages	Disadvantages
Cytology	78-100%	12-85%	High specificity Established technique Minimal sample processing required	High level of expertise required Significant inter-observer variation Low sensitivity (esp. for low grade lesions)
BTA-Stat®	52-93%	24-89%	Rapid point-of-care test	High false positive rate Only approved for surveillance
BTA-Trak®	48-95%	51-79%	Sensitivity comparable to cytology (and better for low grade lesions)	High false positive rate Specialised laboratory required Only approved for surveillance
NMP22 BladderChek®	40-90%	50-85%	High specificity and sensitivity Rapid point-of-care test Approved for both primary diagnosis and surveillance	High false positive rate
NMP22® Test	46-88%	50-91%	High specificity and sensitivity Approved for both primary diagnosis and surveillance	High false positive rate Specialised laboratory required
ImmunoCyt®	62-79%	63-85%	High sensitivity (esp. for lower grade & stage lesions)	Specialised lab and equipment required High level of expertise required Only approved for use in conjunction with cytology
Urovysion®	65-96%	69-96%	High sensitivity and specificity Low false positive rate May identify early sub-clinical neoplastic changes Approved for both primary diagnosis and surveillance	Specialised laboratory equipment required Expensive reagents High level of expertise required Not suited to high-throughput testing

Table 1. Comparison between cytology and commercially available biomarker tests for use in the diagnosis and surveillance of bladder cancer. The broad range of percentages provided for sensitivity and specificity reflect differences in study sizes, assay cut-points, and the grade and stage of tumours studied. [†]Data obtained from references 13 & 24.