Review Article on Expectant Management in Genitourinary Malignancies (Prostate, Bladder, Kidney)

Trends in urine biomarker discovery for urothelial bladder cancer: DNA, RNA, or protein?

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Abstract: Urothelial bladder cancer is a complex disease displaying a landscape of heterogenous molecular subtypes, mutation profiles and clinical presentations. Diagnosis and surveillance rely on flexible cystoscopy which has high accuracy, albeit accompanied by a high-cost burden for healthcare providers and discomfort for patients. Advances in "omic" technologies and computational biology have provided insights into the molecular pathogenesis of bladder cancer and provided powerful tools to identify markers for disease detection, risk stratification, and predicting responses to therapy. To date, numerous attempts have been made to discover and validate diagnostic biomarkers that could be deployed as an adjunct to the cystoscopic diagnosis and long-term surveillance of bladder cancer. We report a comprehensive literature analysis using PubMed to assess the changing trends in investigating DNA, RNA, or proteins as diagnostic urinary biomarkers over a period of 5 decades: 1970–2020. A gradual shift has been observed in research away from protein biomarkers to nucleic acids including different classes of RNA, and DNA methylation and mutation markers. Until 2000, publications involving protein biomarker discovery constituted 87% of the total number of research articles with DNA comprising 6% and RNA 7%. Since 2000 the proportion of protein biomarker articles has fallen to 40%, and DNA and RNA studies increased to 32% and 28%, respectively. Clearly research focus, perhaps driven by technological innovation, has shifted from proteins to nucleic acids. We optimistically hypothesise that, following thorough validation, a clinically useful detection test for bladder cancer based on a panel of DNA or RNA markers could become reality within 5–10 years.

Keywords: Urine; biomarker; trends; bladder cancer; DNA; RNA; protein

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Introduction

Urothelial bladder cancer (UBC) typically presents as painless haematuria, demanding prompt investigation by cystoscopy and upper tract imaging to ascertain diagnosis (1). Non-muscle-invasive bladder cancer (NMIBC, stages: Tis/Ta/T1) comprises 75–80% of new UBC diagnoses, with muscle-invasive disease (MIBC, stage: T2+) comprising the remainder (2). Following initial transurethral resection of tumour (TURBT) and adjuvant intravesical therapy where indicated, long-term surveillance via serial cystoscopy remains an essential mainstay of NMIBC management due to the high incidence of recurrence and a risk of progression to MIBC (1,3,4). Despite cystoscopy being invasive, uncomfortable, expensive and incurring morbidity, no alternative is recommended in current clinical guidelines (1,5).

Following TURBT, tumour grade, TNM stage and other clinico-pathological parameters are used to classify disease according to prognosis and to tailor treatment (1),

but there exists significant heterogeneity within these 'disease states' limiting the applicability of existing classifiers. Given the complex nature of UBC, this is not unexpected; hence, understanding the underlying biology of urothelial carcinogenesis could substantially transform clinical efficiency and patient management. The molecular pathways in bladder cancer, highlighting key pathways of hallmark features leading to urothelial transformation were described in 2005 in the 'pre-genomics' era (6,7), and the more aggressive nature of tumours derived from the basal layer of the urothelium was first considered as early as 2008 (8,9). More recently, DNA and RNA sequencing-based analyses have significantly expanded and detailed these concepts (10-14), with Kamoun *et al.* outlining a consensus molecular classification of MIBC based upon 6 molecular subtypes and associated clinical outcomes (15). Hence, ongoing technological developments in "omics" with concomitant verification via bioinformatics have opened a plethora of possibilities for potential biomarkers. A key output of such research would be the identification of diagnostic molecular markers measurable in urine with high specificity and sensitivity in order to streamline UBC diagnosis and NMIBC surveillance, aid clinical decision-making, and improve cost-effectiveness.

Cystoscopy has been considered as the 'gold standard' for the diagnosis of bladder cancer since its application within an outpatient setting in London in 1984 (16). Subsequent improvements have been made to the method to assist in diagnosis and surveillance viz. blue-light cystoscopy (photodynamic diagnosis) and narrow-band imaging (17-20). Although highly operator-dependent, the overall sensitivity surpasses any other current form of diagnostic modality for the detection of UBC with sensitivity estimated at 85%, with 87% specificity (21).

Novel biomarker discovery has been fuelled by exhaustive research efforts in the last few decades to determine an optimal molecular substrate. A number of urine-based tests have been approved by the US FDA (e.g., NMP22, NMP22 BladderChek Test, BTA TRAK, BTA Stat, UroVysion® and ImmunoCyt), but none are robust enough to facilitate early detection and risk stratification of UBC for routine clinical implementation (22). Instead, urinary cytology remains the most commonly used adjunct to cystoscopy, with high sensitivity to diagnose high-grade disease (sensitivity of 84%), despite its limitations in identifying low-risk cancer (sensitivity 16–48%) (23).

Conceptually, a biomarker would identify targets in the DNA, RNA or protein of tumour cells and be able to distinguish them from normal cells. As urine is in direct contact with the tumour tissue itself, appreciable amounts of protein and genomic material can be found within urine, either in exfoliated UBC cells or as cell-free constituents. Sample collection is inexpensive, painless, and repeatable, and so urine is a logical source for biomarker analysis—a 'liquid biopsy', a simple example of which includes voided urinary cytopathology (VUC) where cells are directly observed microscopically.

In this current review, we have outlined the changing trends over the last five decades in the pursuit of an optimal diagnostic urinary biomarker, focusing on the different classes of biomolecule that can act as a biomarker substrate for UBC- DNA, RNA, protein.

Urine biomarker trends

Searches were performed on Medline and PubMed platforms for available literature using the following keywords: ("urinary bladder neoplasms"[MeSH Terms] OR ("urinary"[All Fields] AND "bladder"[All Fields] AND "neoplasms"[All Fields]) OR "urinary bladder neoplasms"[All Fields] OR ("bladder"[All Fields] AND "cancer"[All Fields]) OR "bladder cancer"[All Fields]) OR "urine"[MeSH Subheading] OR "urine"[All Fields] OR "urine"[MeSH Terms] OR "urines"[All Fields]) AND ("biomarker s"[All Fields] OR "biomarkers"[MeSH Terms] OR "biomarkers"[All Fields] OR "biomarker"[All Fields]). Articles from January 1970 to January 2020 were included with the prerequisites of being in the English language, with available abstracts. Review articles, letters, editorials, or comments were excluded. Journal articles were included if the reported studies were performed on urine. Biomarkers were categorized as DNA/genomic, RNA/transcriptomic or protein.

Data were collated from articles regarding: the biomarker assay type, the technique utilized for analysis, assessment utility, and where available—associated sensitivity, specificity, positive predictive value and negative predictive value.

The database search identified 1929 articles. Two additional searches were created using the following: "urine biomarker DNA" and "urine biomarker RNA", resulting in 301 and 224 results, respectively. 843 articles were reviewed (*Figure 1*) that reported either DNA, RNA or protein biomarkers (*Figure 2A*), with a breakdown of classes of RNA or DNA marker and their discovery observed over 5 decades (*Figure 2B*).

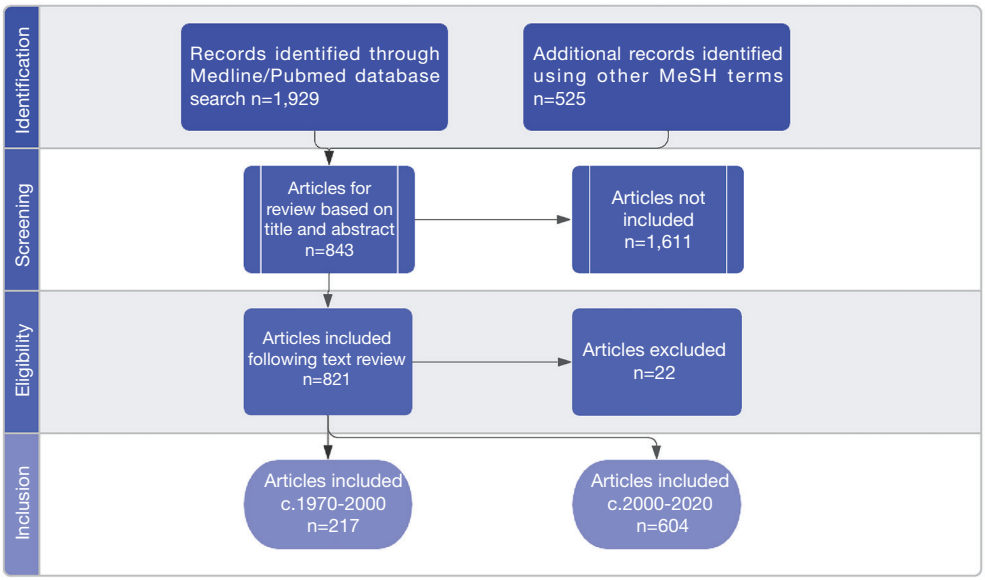

Figure 1 PRISMA flow diagram detailing the studies retrieved from database search, screened, and included for review.

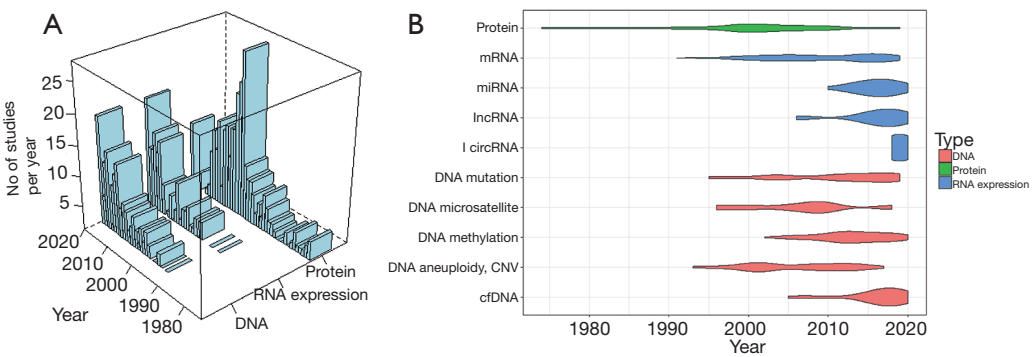

Figure 2 Detailed breakdown of publications according to molecular subtype per decade. (A) Number of studies per year that investigated DNA, RNA expression or protein as urinary biomarker. A gradual increase in trends of DNA and RNA biomarker discovery is observed with a shallow decline in protein studies. (B) Illustration above shows a breakdown of studies investigating different molecular substrates to identify diagnostic and prognostic urine biomarkers for bladder cancer. mRNA, messenger RNA; miRNA, microRNA; lncRNA, long non-coding RNA; circRNA, circular RNA; cfDNA, cell-free DNA.

Proteins

Early protein biomarker research studies were limited to antibody-based measurements of one, or a small number of, biomarker candidates e.g., Carcinoembryonic antigen (CEA) (24), tissue plasminogen activator (TPA) (25), fibronectin (26). The advent of "proteomics" (2D-electrophoresis in the 1970s and protein identification by mass spectrometry in the 1980s) enabled biomarker discovery via comparison of the protein composition of cell lines and tumour and non-tumour tissues and urine e.g., (27,28). Since 2000 there have been enormous improvements in the speed, accuracy and sensitivity of mass spectrometers and the combination of two-dimensional fractionation of tryptic peptides with mass spectrometry (or "shotgun proteomics") has enabled more in-depth proteomic analysis allowing, for example, identification and quantitation of >1,500 proteins in human urine (29,30). In the last decade targeted peptide mass spectrometry ("MRM") and multi-plex immunoassay platforms have enabled the measurement of panels of protein biomarker

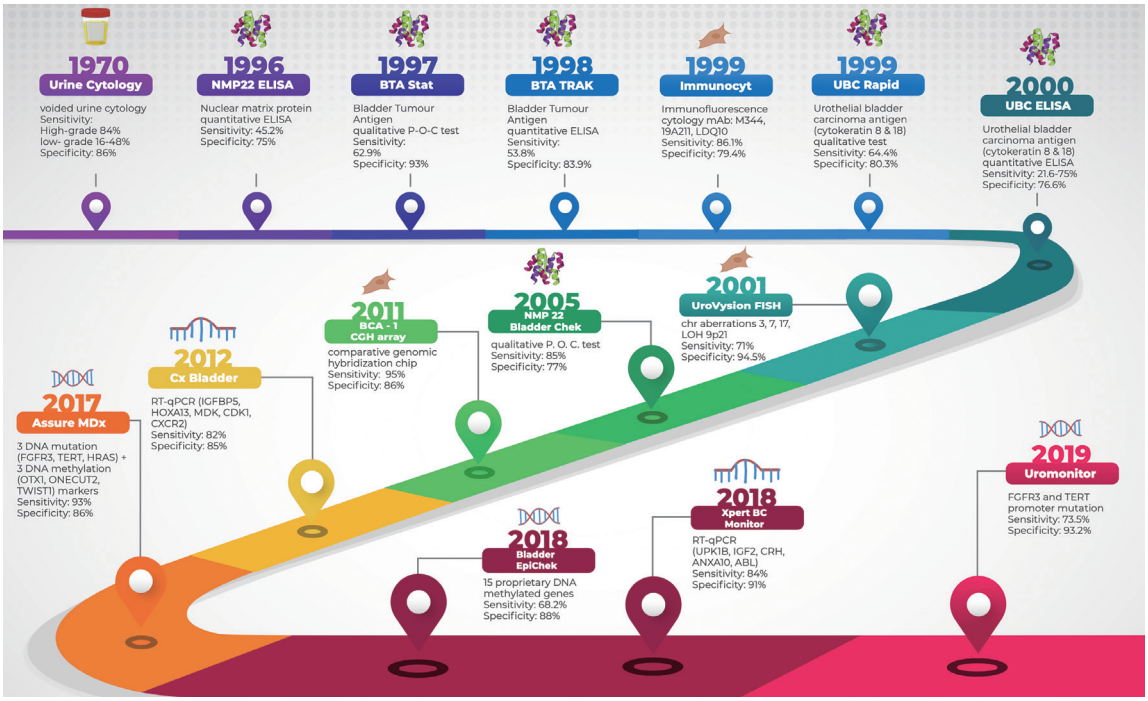

Figure 3 Evolution of US FDA-approved (NMP22 ELISA, NMP22BladderChek, BTAstat, BTA TRAK, Immunocyt/uCyt+, UroVysion) and other commercially available biomarkers with their sensitivity and specificity (99-110). This figure has been designed using resources from Freepik.com.

candidates derived from transcriptomic and proteomic analyses (31).

From the 1970s to 2000s, 208 studies evaluated the utility of urinary proteins as biomarkers. We found 25 studies investigating the potential use of urinary CEA as an adjunct to cystoscopy or as a stand-alone diagnostic biomarker (24,32-55). Twelve studies investigated survivin, a protein which inhibits apoptosis allowing tumour cell survival (56-68). Fragments of cellular cytoskeletal proteins cytokeratins 8, 18, 19 and 20 (69) have also been found in urine of UBC patients, leading to the development of Urothelial Bladder Carcinoma- antigen assay (UBC) (66,70-79) and Cyfra21.1 (80-91). Vascular endothelial growth factor (VEGF) has been investigated in 10 studies (59,64,86,92-98). Several proteins have been commercialised as bladder cancer detection tests (*Figure 3*) although only NMP22 and BTA measured as soluble proteins in urine, and immunocyte which measures mucins and a glycoform of CEA on exfoliated cells have gained FDA approval. NMP22 and BTA can be measured quantitatively using Enzyme linked immunosorbent assay (ELISAs) or qualitative point-of-care tests, validated in 117, 97 and 43 studies, respectively.

Combining protein biomarkers into multi-analyte tests has largely failed to increase sensitivity and specificity although it remains possible that tumour specific variants of proteins (e.g., novel splice variants or glycoforms) could offer improved performance. Though a large proportion of bladder cancer biomarker discovery research has focused on proteins, developments in genomics, epigenetics and transcriptomics have shifted the research towards nucleic acid biomarkers (discussed below). This is evident in the continued decline in the protein biomarker studies reported in the last decade.

DNA and genomics

Game changing technological developments such as polymerase chain reaction (PCR) in the 1970s, microarrays in the 1980s and massively parallel sequencing (next generation sequencing or NGS) in the 2000s have facilitated biomarker research, characterization of cellular and molecular pathways, and provided targets for novel therapeutics and drug discovery (10,11). The concept of

"liquid biopsy" fostered the analysis of DNA in plasma and urine, in studies involving methylation, mutation, and copy number variations (CNV) (111). Due to the molecular heterogeneity of UBC, multiplexed genomic profiling of DNA is likely to prove more useful than individual markers.

DNA: copy number changes and microsatellite instability

Loss of chromosome 9p, 9q and 17 (112,113) are common early events in bladder cancer and a large fraction of the entire genome undergoes copy number changes in high-grade disease (114-116). These phenomena are utilised by several commercially available bladder cancer tests; UroVysion uses fluorescence *in situ* hybridization (FISH) to detect aneuploidy for chromosomes 3, 7, 17, and loss of 9p21 in exfoliated cells (117-122) and BCA-1 uses array comparative genomic hybridisation to analyse genome wide copy number changes (109,123,124) (*Figure 3*). More recently, microarrays and next generation sequencing have been used to study copy number changes in urine cell-free and pellet DNA (116,125-127). In the 1990s microsatellite instability markers were widely researched for diagnosis but this has now ceased (128,129).

DNA: mutations

Tumour DNA from urine can be extracted following centrifugation (cell pellet DNA, cpDNA) or as fragmented cell-free DNA (cfDNA) in the supernatant (130,131); both forms are admixed with a large background of normal DNA. However, using highly sensitive analytical approaches such as digital droplet PCR (ddPCR) and next-generation sequencing (NGS), it is possible to detect cancer-associated mutations at very low levels in urine.

Two distinct pathways have been recognized that encourage the development of NMIBC or MIBC, by activation of MAPK-PIK3 pathway or disruption of cell cycle regulators (132,133). 11 studies have found mutations in *FGFR3*, *RAS*, *PIK3CA* to translate phenotypically to NMIBC (99,134-144), whereas more aggressive MIBC states are generally characterized by mutations in *TP53*, *CDKN2A*, *MLL* and *ERBB2* (n=6 studies) (12,145-150). 11 studies were found on *TERT* promoter mutation; which has been observed in the early stages of tumour development occurring in 74% of incident and recurrent cases, and is also independently related to poor survival outcomes reflecting their potential usage as prognostic markers (151-155). The analyses of multiple DNA mutations (19 studies) have provided better overall sensitivity and specificity for the detection of NMIBC by use of next generation sequencing (99,115,135,141-145,149,156-159).

DNA: methylation

DNA methylation status can be readily assessed in bisulphite converted cpDNA and cfDNA through quantitative methylation specific PCR (QMSP-PCR), pyrosequencing, NGS and/or ddPCR (*Table 1*). We found 54 studies that investigated methylation markers in urine for UBC detection. An assay based on 15 methylation markers has been commercialised (*Epicheck*) and another using a combination of 3 DNA methylation (*OTX1*, *ONECUT2*, *TWIST1*) and 3 mutation markers (*HRAS*, *FGFR3*, *TERT*)-*Assure MDx* functional biomarker (*Figure 3*).

RNA and transcriptomics

Expression profiling with microarrays studies and more recently RNA sequencing has identified numerous overexpressed transcripts in bladder cancer. Most of this work focused on protein coding messenger (m)RNAs although additional classes of RNA [micro(mi)RNA, long non-coding (lnc)RNA and circular (circ)RNA] have come to the fore recently (described below). We found 61 urine mRNA biomarker studies, mostly using RT-qPCR to measure specific gene products. The commercially available Cx bladder test analyses a panel of 5 genes that are overexpressed in bladder cancer by RT-qPCR.

miRNA

Micro RNAs are short non-coding RNAs that impose post-translational effects by binding onto the 3' untranslated region (UTR) of their target mRNA, to regulate gene expression. miRNA-altered gene expression has been found to initiate and promote tumour progression, making them potential diagnostic and prognostic indicators. MiRNAs are stable and detectable in body fluids, including urine (201,202). With the aid of computational biological methods, identification and stratification of miRNA transcripts can be scrutinized for expression levels. Subsequent analysis can be validated using quantitative reverse transcriptase polymerase chain reaction (qRT-PCR), ddPCR, microarray, pyrosequencing or NGS. Since their discovery (203), multiple studies have been carried out to research this entity in urine (*Table 2*).

Table 1 Studies involving DNA methylation markers and their characteristics

Year	Target genes	Method	Sample	Sensitivity	Specificity	Ref
2002	*RARbeta, DAPK, E-cadherin, p16, p15, GSTP1,* and *MGMT*	qMSP-PCR	Urine, tissue	90.9	76.4	(160)
2006	*APC, ARF, CDH1, GSTP1, MGMT, CDKN2A, RARbeta2, RASSF1A,* and *TIMP3*	RT-PCR	Urine	82	96	(161)
2006	*Wnt-antagonist genes (sFRP-1, sFRP-2, sFRP-4,* and *sFRP-5, Wif-1,* and *Dkk-3)*	qMSP-PCR	Urine, tissue	77.2	66.7	(162)
2006	*RASSF1a, E-cad* and *APC*	qMSP-PCR	Urine	69	60	(163)
2007	*SALL3, CFTR, ABCC6, HPR1, RASSF1A, MT1A, RUNX3, ITGA4, BCL2, ALX4, MYOD1, DRM, CDH13, BMP3B, CCNA1, RPRM, MINT1,* and *BRCA1*	qMSP-PCR	Urine, tissue	91.7	87	(164)
2007	*p14ARF, p16 CDKN2A, STAT-1, SOCS-1, DR-3, DR-6, PIG-7, BCL-2, H-TERT, BAX, EDNRB, DAPK, RASSF-1A, FADD, TMS-1, E-CADHERIN, ICAM-1, TIMP-3, MLH-1, COX-2*	qMSP-PCR	Urine, tissue	81.1	100	(165)
2009	*CDH1, FANCF, LOXL1, LOXL4, p16INK4, SFRP1, SOX9, TIG1, TIMP3,* and *XAF1*	qMSP-PCR	Cell lines, tissue, urine	91.7	87	(166)
2009	*BCL2, hTERT,* and *DAPK*	qMSP-PCR	Urine, tissue	76	98	(167)
2010	*GDF15, HSPA2, TMEFF2,* and *VIM*	qMSP-PCR	Cell lines, tissue, urine	94	90	(168)
2010	*TWIST1* and *NID2*	qMSP-PCR	Cell lines, tissue, urine	90	93	(169)
2010	*E-cadherin, p16, p14,* and *RASSF1A*	qMSP-PCR	Urine, tissue	85	75–79	(170)
2011	*APC, DAPK, E-cadherin, hMLH1, IRF8, p14, p15, RASSF1A, SFRP1* and *SOCS-1*	qMSP-PCR	Urine	86.7	94.7	(171)
2011	*MYO3A, CA10, SOX11, NKX6-2, PENK,* and *DBC1*	qMSP-PCR	Urine	81–85	95–97	(172)
2011	*FGFR3, PIK3CA, TP53, HRAS, NRAS* and *KRAS for mutations; methylation- APC, ARF, DBC1, INK4A, RARB, RASSF1A, SFRP1, SFRP2, SFRP4, SFRP5* and *WIF1*	PCR, MethyLight	Urine, tissue	62	100	(136)
2012	*BCL2, CDKN2A* and *NID2*	Amplicon, RT-PCR	Urine	80.9	86.4	(173)
2012	*EOMES, HOXA9, POU4F2, TWIST1, VIM,* and *ZNF154*	–	Urine	82–89	94–100	(174)
2012	*RAR-β2, hyaluronidase activity*	qMSP-PCR	Urine	65, 89	89.7, 90.5	(175)
2012	*PCDH17* and *TCF21*	–	Cell lines, tissue, urine	60	100	(176)
2012	*ZNF154, HOXA9, POU4F2, EOMES, ACOT11, PCDHGA12, CA3,* and *PTGDR*	qMSP-PCR	Urine	84	96	(177)
2013	*FGFR3, CpG islands (CGI)*	SNaPshot Multiplex kit	Urine	79	77	(178)

Table 1 (*continued*)

Table 1 (*continued*)

Year	Target genes	Method	Sample	Sensitivity	Specificity	Ref
2013	*1303 CpG sites; SOX1, TJP2, VAMP8* and *SPP1*	Illumina golden gate methylation assay, pyrosequencing 96HS	Urine, tissue	94.3	97.8	(179)
2013	*TWIST1* and *NID2*	qMSP-PCR	Urine, tissue	88.8		(180)
2014	*ARF, TIMP3, RAR-β2, NID2, CCNA1, AIM1, CALCA* and *CCND2*	qMSP-PCR	Urine, tissue	72.7	70	(181)
2014	19 genes incl. *VGF*	qMSP-PCR	Urine, tissue	40	95	(182)
2014	7 genes	MethyLight and pyrosequencing assays	Urine	80	84–88	(183)
2014	6 markers	Bisulphite- treated RT-PCR	Urine	80	97	(184)
2014	*TWIST1* and *NID2*	qMSP-PCR	Urine	75	71	(185)
2015	*ZNF671, IRF8* and *sFRP1*	Bisulphite pyrosequencing	Urine, tissue	42–48	96.2	(186)
2015	*TWIST1* and *NID2*	qMSP-PCR	Urine	67	69	(187)
2016	*HS3ST2, SLIT2* and *SEPTIN9*	qMSP-PCR	Urine	97.6	84.8	(188)
2016	*p14ARF, RUNX3, RARβ, DAPK,* and *HPP1*	qMSP-PCR	Urine	98.2	88.9	(189)
2016	*EOMES, GDF15, NID2, PCDH17, POU4F2, TCF21,* and *ZNF154*	qMSP-PCR	Urine	90	93.96	(190)
2017	*Uromark*	NGS	Urine	98	97	(191)
2017	*p14ARF, p16INK4A, RASSF1A, DAPK, APC*	qMSP-PCR	Urine	–	–	(192)
2017	*Assure MDx: mutation of FGFR3, TERT* and *HRAS,* and *methylation of OTX1, ONECUT2* and *TWIST1*	–	Urine	93	86	(110)
2018	*CDH13, CFTR, NID2, SALL3, TMEFF2, TWIST1,* and *VIM2)*	Bisulphite- treated pyrosequencing	Urine	96	40	(193)
2018	*VIM, CDH1, SALL3, TMEFF2, RASSF1A, BRCA1, GDF15,* and *ABCC6*	–	Urine	89	74	(194)
2018	*DDR*	RT-PCR, IHC	Urine	52	91	(195)
2019	6 markers *(GynTect)*	MSP- PCR	Urine	60	96.7	(196)
2019	*HOXA9, ONECUT2, PCDH17, PENK, TWIST1, VIM* and *ZNF154*	MSP-PCR	Urine, tissue	AUC of 0.894 and 0.851		(197)
2019	*BladderEpiChek*	RT-qPCR	Urine	LG: 46.1; HG: 83.3	86.3	(198)
2019	*GHSR/MAL*	PCR	Urine	92	85	(199)
2020	*HOXA9, PCDH17, POU4F2,* and *ONECUT2*	–	Urine	90.5	73.2	(200)

lncRNA

LncRNAs are long RNA molecules that do not encode proteins. They may serve as regulators of gene expression and recent high throughput profiling studies have shown dysregulation of lncRNAs to promote oncogenesis. lncRNAs found in urine have been associated with UBC (*Table 3*) which merits further exploration to uncover their functional roles in tumorigenesis. Urothelial-carcinoma-associated 1 (UCA-1) lncRNA is a potential biomarker that is upregulated in the presence of UBC in proportion to the grade of disease (226,233).

circRNA

An emerging novel class of RNAs are circular RNAs (circRNA). These are single-stranded RNAs that are covalently linked through backsplicing events to form a closed continuous loop. They are stable, immune to degradation by RNAases, and can be found in urine (*Table 3*) (234). Emerging evidence indicates their role in regulating gene expression by "sponging" miRNAs (235). Recent studies profiling circRNA in tumour tissue (236), demonstrate that their relative expression levels are indicative of tumour grade and stage (231,237), and involvement in urothelial carcinogenesis (238), providing insights into a fascinating new genre of biomarkers.

Conclusion and future prospects

Over the course of 5 decades, technological advances have transformed biomarker discovery from immunoassays for single protein biomarkers through to whole genome sequencing and accessing new classes of modifications and biomolecules. Challenges remain to detect even the slightest amount of tumour DNA from large amount of background normal DNA. Whereas the prospects of available urinary biomarkers are generally promising, an element of bias in regard to sample selection still exists i.e., performance evaluation of the test relies on the prevalence of underlying disease in specific cohorts. There may also be resistance from medical practitioners to change practice until biomarker tests match or exceed the performance of cystoscopy (even though cystoscopy itself is imperfect).

Additionally, bladder cancer research itself faces significant bias in terms of funding opportunities. It has been reported that in 2017/18, £4.5 million were spent on bladder cancer by Cancer Research UK, compared to £22.1 million for prostate cancer and £7.3 million for renal cancer (239). Many biomarkers exist with the potential to transform bladder cancer detection and even provide personalized medicine, but due to poor research funding and lack of level I evidence, uptake into clinical practice is hesitant.

Large-scale multicentre prospective clinical trials are required to validate the performance of promising biomarkers that have demonstrated potential clinical utility: sensitivities and specificities to match or exceed cystoscopy, the capability to be multiplexed with high throughput, and accompanied by limited impact on resources (22). Given the funding challenges across all of bladder cancer research, such studies have not yet been undertaken. Hence, the current generation of highly promising biomarkers remain 'on the bench', both physically and metaphorically. Notwithstanding, a role of cystoscopy will be ever present for the diagnosis of other conditions of the lower urinary tract.

As we enter the 2020s, further diagnostic biomarker classes are emerging. For example, the profiling of the urinary bladder microbiome to investigate causal relationships with UBC. In other cancer settings, the microbiome possesses mutagenic properties that can initiate tumorigenesis by way of chronic insult, such as in the case of *Helicobacter pylori* infection and gastric cancer (240). Recent studies have reported an abundance of *Acinetobacter spp.* from patients with HR-NMIBC (241-243). The preliminary studies suggest that there is potential in investigating the urinary microbiome for early screening of UBC, but much remains to be discovered.

Likewise, extracellular vesicles (EV)—small spherical membrane vesicles secreted by all cells, cargo nucleic acids or proteins, and are the responsible machinery for intercellular communication (244). They are especially important in cell-cell signalling, extracellular-matrix (ECM) remodelling, and distant spread/ metastasis when secreted by cancer cells as exosomes (245). Exosomes act as vehicles for the transport of mRNA, miRNA, lncRNA, tumour DNA and protein to build and nurture tumour micro-environment (TME) for development and progression of cancer. EVs have been isolated from urine supernatant through ultracentrifugation and characterized by MS (246), nanoparticle tracking (247), immunoaffinity-capture, microfluidic-filtration (248). Whichever method is utilized, the challenge is to obtain sufficient quantity of undiluted EV for analysis, hence there is no consensus on one set technique. EVs have gained attention in the

Table 2 Studies involving miRNA detection in UBC and their characteristics

Year	Target	Method	Sample	Sensitivity	Specificity	Notes	Ref
2013	methylation of miR-137, miR-124-2, miR-124-3, and miR-9-3	Taqman array, bisulfite pyrosequencing	Bca cell line, urine, tissue	81	89	Ectopic expression of silenced miRNAs in BCa cells suppressed growth and cell invasion.	(204)
2013	6 miRNA diagnostic (D); 2 miRNA prognostic (P)	TaqMan Human MicroRNA Array, miRCURY LNA qPCR system	Urine	D: 84.8; P: 84.95	D: 86.5; P: 74.14	–	(205)
2014	miRNA-99a and miRNA-125b	RT-qPCR	Urine	86.7	81.1	MicroRNA-125b alone exhibited a sensitivity of 81.4%, a specificity of 87.0%	(206)
2014	cell- free oncogenic miR-106b-25 cluster: (miR-106b, miR-93 and miR-25)	RT-qPCR	Urine	76.8	72.4	–	(207)
2015	miRNA-96	RT-qPCR	Urine	72.3	88.9	–	(208)
2015	miR-96, miR-182, miR-183, miR-200c, miR-21, miR-141 and miR-30b	Taqman RT-qPCR	Urine, bladder washings	–	–	miR-182 is higher in cytology specimens from high-grade UCC patients as compared to normal controls	(209)
2015	miRNA-141 and miRNA-200b	Cell invasion assay, WB, RT-qPCR	Urine	88	43.4	Invasion and EMT	(210)
2015	miRNAs (miR-21, and let-7a) + SNP let-7 family of miRNAs	Mach-Zehnder interferometer-miRNA detection system	Urine	–	–	Point-of-care testing	(211)
2016	miR-155	RT-qPCR	Urine	80.2	84.6	–	(212)
2016	miR 16, miR 21, miR 34a, miR 99a, miR 106b, miR 126, miR 129, miR 133a, miR 145, miR 200c, miR 205, miR 218, miR 221/222, miR 331	RT-qPCR	Urine	88	48	–	(213)
2016	miR-125b, miR-30b, miR-204, miR-99a, and miR-532-3p	Microarray; single target-qPCR	Urine	miR-125: 95.65, miR-99a: 82.61	miR-125: 59.26, miR-99a: 74.07	–	(214)
2016	46 microRNAs	TaqMan low density arrays	Urine	87	100	–	(215)
2017	miR-140-5p and miR-92a-3p	RT-qPCR	Urine	55	84	–	(216)

Table 2 (*continued*)

Table 2 (*continued*)

Year	Target	Method	Sample	Sensitivity	Specificity	Notes	Ref
2017	miR-7-5p, miR-22-3p, miR-29a-3p, miR-126-5p, miR-200a-3p, miR-375, and miR-423-5p	Deep-sequencing; RT-qPCR	Urine	82	96	–	(217)
2018	miR-9-3, miR-124-2, miR-124-3, and miR-137	Methylation-bisulphite pyrosequencing; M-score	Urine	61.5	74	–	(218)
2018	miR-31-5p, miR-93-5p and miR-191-5p	Affymetrix miRNA microarrays; RT-qPCR	Urine	81	70	–	(219)
2018	miR-30a-5p, let-7c-5p and miR-486-5p	NGS, RT-qPCR	Urine	–	–	AUC 0.70	(220)
2019	cell-free microRNA: miR-6124 to miR-4511	Microarray, RT-qPCR	Urine	91.5	76.2	–	(221)
2018	let-7c, miR-135a, miR-135b, miR-148a, miR-204, miR-345	Taqman™ Human MicroRNA Array	Urine	–	–	AUC 92.9%	(222)
2018	miR-141, miR-10b, miR-34b and miR-103	RT-qPCR	Urine	75	63.5	–	(223)
2019	miR-146b-5p, miR-155-5p, miR-138-5p, miR-144-5p, and miR-200a-3p + EV	Microarray, RT-qPCR	Urine, tissue	–	–	P<0.05 between tissues from MIBC and NMIBC tumours	(224)
2020	microRNA-192 + B ultrasound	RT-qPCR	Urine	miR-192: 76.7, miR + BUSS: 93.2	miR-192: 78, miR + BUSS: 76.7	–	(225)

Table 3 Study characteristics of lncRNA and circRNA found in urine for diagnosis of UBC, with their sensitivity and specificity

Year	RNA	Gene	Analytic approach	Sample	Sensitivity	Specificity	Notes	Ref
2014	lncRNA	*UCA-1*	RT-PCR	Urine, T24 cell line	79.5	79.7	High grade disease (G2-G3) sensitivity =84.09%	(226)
2015	lncRNA	*UCA-1*	RT-PCR	Urine	70	70.7	Limited role in follow-up recurrence tumours	(227)
2015	lncRNA	*UCA1*	Hybridization assay; Nanoassay RT-qPCR	Urine	92.1	93.3	–	(228)
2017	lncRNA	*H19*	RT-PCR	Urine	90.5	74.1	–	(229)
2018	lncRNA (cell free)	*uc004cox.4 GAS5*	microarray, RT-qPCR	Urine	84.5	78.2	–	(230)
2020	circRNA	*Hsa_circ_0137439*	microarray, RT-qPCR	Urine	87.93	80.06	Independent prognostic predicator of RFS and OS; Knockdown contributed to inhibition of cell proliferation and migration via hsa_circ_0137439/miR-142-5p/MTDH axis	(231)
2020	lncRNA	*UCA1-201, HOTAIR,* *HYMA1, MALAT1*	RT-qPCR	Urine	95.7	94.3	–	(232)

second-half (2015–2020) of the last decade and following a standardized technique for analysis, they could be revolutionary for the forthcoming era.

And so, having demonstrated the evolution of research into the biomolecule substrates for urinary diagnostic biomarkers from the 1970s to date, from proteins to DNA/RNA, the field continues to expand. Notwithstanding, clinically relevant and acceptable sensitivities and specificities at affordable costs from the current generation of diagnostic urinary biomarkers are very much within reach.

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Footnote

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