The emerging role of extracellular vesicles as biomarkers for urogenital cancers

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Abstract | The knowledge gained from comprehensive profiling projects that aim to define the complex genomic alterations present within cancers will undoubtedly improve our ability to detect and treat those diseases, but the influence of these resources on our understanding of basic cancer biology is still to be demonstrated. Extracellular vesicles have gained considerable attention in past years, both as mediators of intercellular signalling and as potential sources for the discovery of novel cancer biomarkers. In general, research on extracellular vesicles investigates either the basic mechanism of vesicle formation and cargo incorporation, or the isolation of vesicles from available body fluids for biomarker discovery. A deeper understanding of the cargo molecules present in extracellular vesicles obtained from patients with urogenital cancers, through high-throughput proteomics or genomics approaches, will aid in the identification of novel diagnostic and prognostic biomarkers, and can potentially lead to the discovery of new therapeutic targets.

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Introduction

Urogenital cancers—cancers of the reproductive and renal organs—are major causes of morbidity and mortality worldwide.[1,2] The multistage, stochastic and heterogeneous nature of these malignancies, resulting from genetic and epigenetic modifications, poses a fundamental challenge to monitoring. Although surgical treatment and chemotherapy for urogenital cancers have improved in the last decade, the prognoses for these diseases remain poor, as existing tests are not sufficiently sensitive or specific to diagnose urogenital cancers at early stages, and none has been shown to significantly decrease overall mortality. Current diagnostic procedures include general examinations and biopsies, such as image-guided prostate biopsy,[3] cystoscopy and transurethral resection of the bladder,[4] nephrectomy and percutaneous renal tumour biopsies,[5] all of which lack sensitivity and can be associated with significant health complications (for example, biopsies are invasive procedures associated with bleeding and risk of infections). Moreover, the location of urogenital cancers deep within the pelvic region makes them hard to access. Thus, in the absence of early symptoms, cancers are diagnosed at an advanced stage, by which time patients have poor outcomes and tumours have often metastasized.

Extracellular vesicles have gained considerable attention in the past 10 years as potential sources for biomarker discovery. These small (40–5000 nm diameter) membrane-bound vesicles are categorized into exosomes, microvesicles or ectosomes, apoptotic bodies[6–10] or Golgi vesicles[11] on the basis of their size, origin, morphology and mode of release. Well-known for biological effects, such as signalling and transfer of cargo, extracellular vesicles are secreted under various pathophysiologic conditions into the extracellular environment by a variety of cell types, promoting tumour progression, survival, invasion and angiogenesis,[12–17] as well as influencing the immune response, cell-to-cell communication, extracellular matrix degradation, coagulation, stem-cell renewal, cardiovascular functions and resistance to drugs (Figure 1).[18–30]

Surprisingly, the biomolecular cargo of extracellular vesicles is stable in biological fluids and protected against exogenous RNases and proteases, owing to its encapsulation within membrane vesicles,[23,31,32] or association with RNA-binding or DNA-binding proteins[33–35] or lipoprotein complexes.[36,37] Thus, extracellular vesicles might be stable under adverse physical conditions, such as extremes in pH, long-term storage and multiple freeze–thaw cycles,[33,38] making them an appealing source for biomarker development.

Several reports indicate that cancer cells release more extracellular vesicles than normal cells,[17,39,40] and that the biomolecular cargo (that is, proteins, nucleic acids and lipids) is reflective of the cell of origin.[41,42] Consequently, knowledge about the content of extracellular vesicles derived from tumour cells with differing stages of aggression could be used to establish new diagnostic approaches using patient-derived vesicles from body fluids. The detection of biomarkers in body fluids has major advantages over the use of tissue markers, which most often require invasive biopsies that can be difficult to perform and potentially dangerous. Urine-based

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Key points

- Extracellular vesicles are small (40–5,000 nm diameter) membrane-bound vesicles that can be categorized into exosomes, microvesicles and apoptotic bodies according to their size, origin, morphology and mode of release
- Whereas the generation of exosomes involves endocytosis, formation of multivesicular bodies and subsequent membrane fusion, microvesicles are produced by membrane budding and apoptotic bodies result from membrane blebbing during apoptosis
- Over the past 10 years, various methodologies for the effective isolation of extracellular vesicles have been developed, including centrifugation, affinity capture, precipitation and the use of microfluidic devices
- Extracellular vesicle cargo is thought to reflect the cell-type of origin, suggesting it could be a promising source for the discovery of novel biomarkers

tests, in particular, could offer attractive approaches for large-scale screening, as large amounts of urine can be collected longitudinally. Ultimately, discriminating between cargoes associated with extracellular vesicles in body fluids using proteomic and genomic profiling approaches could provide insight into disease staging. An important first step is to develop sensitive, rapid and highly effective strategies to enable the collection of extracellular vesicles, and to adapt standardized procedures for routine clinical diagnostic application.

In this Review, we provide a comprehensive overview of the roles of extracellular vesicles in the most common urogenital cancers (prostate, kidney and bladder). This includes a detailed overview of the current knowledge of the different classes of extracellular vesicles, their biogenesis, potential biological functions and available technologies for isolation and downstream analyses. Existing knowledge regarding the cancer-specific biology of extracellular vesicles, and their potential use as vehicles for biomarker discovery, are reviewed and discussed.

Biogenesis of extracellular vesicles

Our current understanding of extracellular vesicles suggests that they comprise a heterogeneous population of exosomes (40–200 nm diameter), microvesicles (also known as ectosomes, 50–1,000 nm) and apoptotic bodies (50–5,000 nm). The formation of exosomes and microvesicles is proposed to occur via two distinct pathways, while apoptotic bodies arise as a consequence of indiscriminate membrane blebbing during apoptosis. Golgi vesicles could also be considered as part of the extracellular vesicle populations,[11] where their existence in body fluids might be representative of distinct disease states. Clearly, an enhanced knowledge of the different mechanisms that drive the biogenesis of these subpopulations could help to identify their utility as diagnostic and therapeutic modalities. Furthermore, in spite of their importance in cellular physiology and disease, little is known about the release of extracellular vesicles and how it is precisely regulated.

Exosomes and microvesicles

The formation of exosomes occurs via endocytosis and internalization of cell-surface receptors into early endosomes. From these structures, proteins and lipids are selectively recruited into recycling endosomes, or targeted for lysosomal degradation by ubiquitylation and ubiquitin-dependent interactions with endosomal sorting complexes required for transport (ESCRT-0, ESCRT-I and ESCRT-II).[43] Alternatively, they can proceed towards a late-endosomal pathway, dependent on multivesicular bodies (MVBs), which is regulated by a ubiquitin-independent recruitment mechanism, for exosome sorting.[44,45] In this pathway, ALIX (ALG-2-interacting protein X, a protein that interacts with ESCRT-III) can bind directly to exosomal

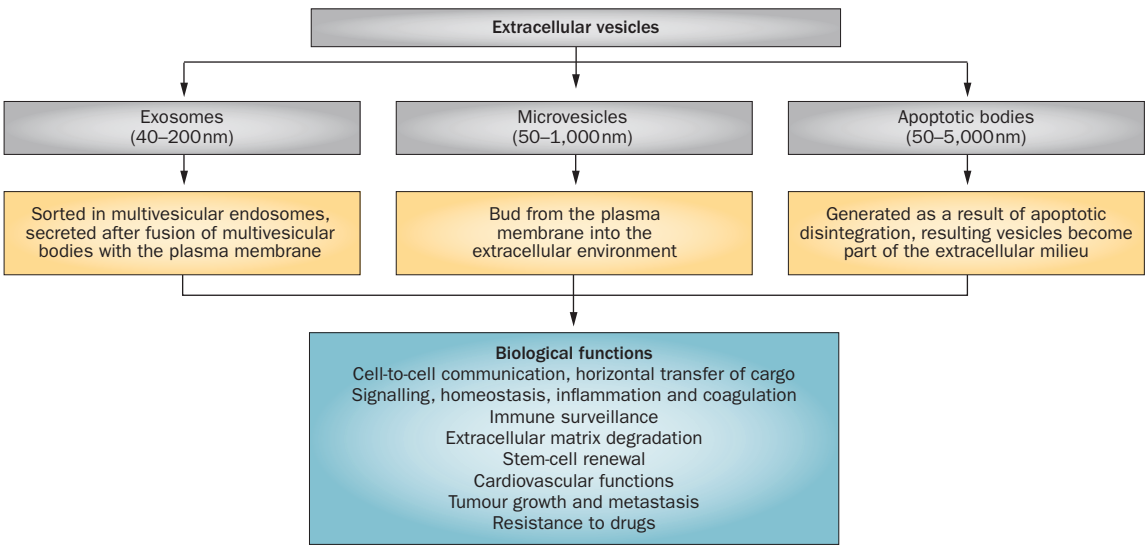

Figure 1 | Classes of extracellular vesicles. Extracellular vesicles comprise a heterogeneous mixture of exosomes, microvesicles and apoptotic bodies. The biogenesis of these three subtypes differs: microvesicles bud directly from the plasma membrane, whereas exosomes are formed by endocytosis and the subsequent formation of multivesicular bodies, and apoptotic bodies are formed as a consequence of apoptotic disintegration. Extracellular vesicles regulate numerous biological functions, such as cell-to-cell communication and horizontal transfer of cargo, and have been implicated in a number of biological pathways.

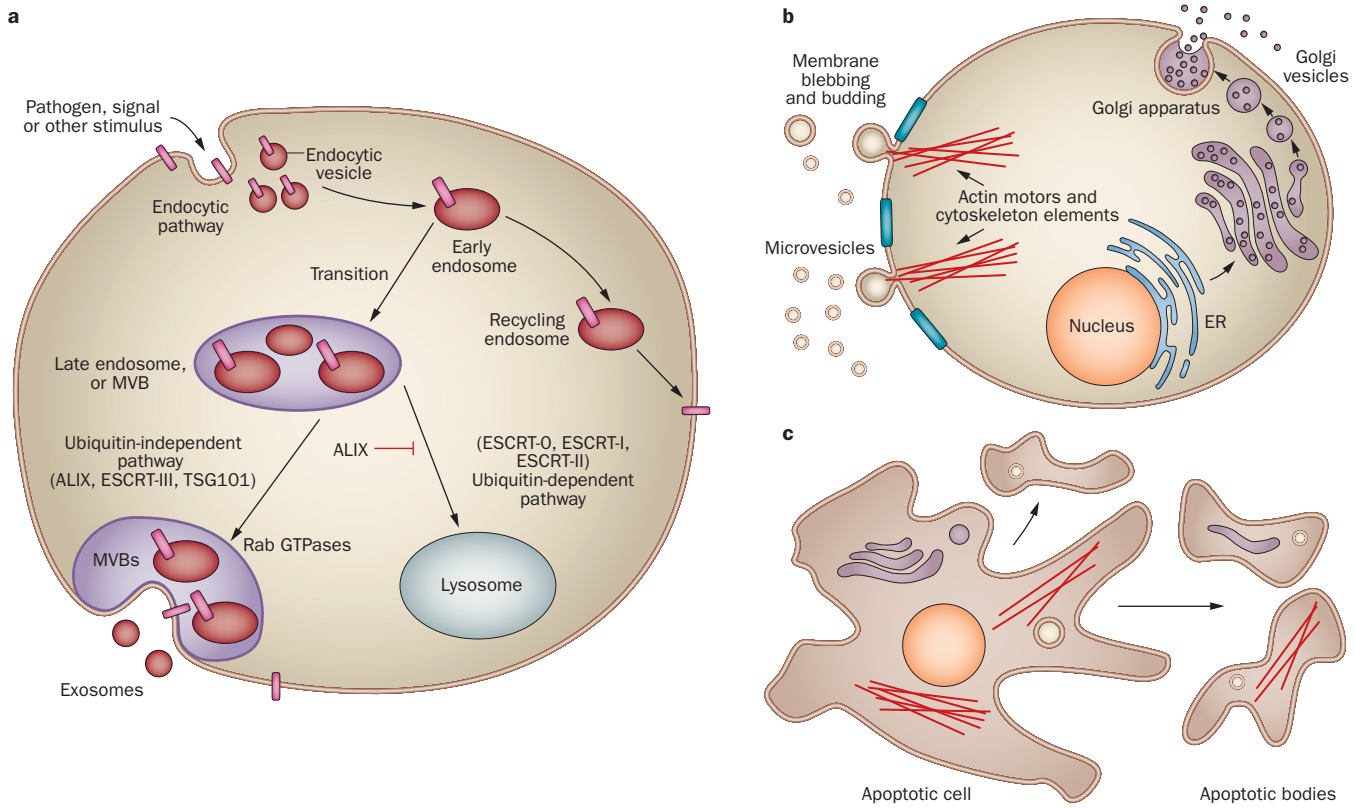

Figure 2 | Extracellular vesicle biogenesis. Extracellular vesicles originate through different mechanisms. **a** | Exosomes initiate as intraluminal vesicles that are formed by endocytosis in response to pathogens, ligands or other stimuli; these endocytic vesicles mature to early endosomes, and then into late endosomes, or MVBs. Following the ubiquitin-dependent interactions with ESCRT complexes, MVBs can be sorted for lysosomal degradation or they can fuse with the plasma membrane and be released as exosomes. ALIX binds to MVB cargo, preventing lysosomal degradation and favouring exosomal release. Rab GTPases regulate MVB fusion with the plasma membrane and release of exosomes. **b** | Microvesicles are formed by the outward budding and fission of plasma membrane lipid microdomains, which is controlled by regulatory proteins and cytoskeleton elements, that promote membrane curvature at ceramide-enriched domains (blue bars), resulting in microvesicle budding. After synthesis in the ER, protein cargo is transported to the Golgi apparatus, modified and packaged into small vesicles secreted as transport Golgi vesicles. **c** | Cells undergoing apoptotic disaggregation produce large membrane blebs, known as apoptotic bodies or apoptosomes. Abbreviations: ALIX, ALG-2-interacting protein X; ER, endoplasmic reticulum; ESCRT, endosomal sorting complexes required for transport; MVB, multivesicular body; TSG101, tumour susceptibility gene 101 protein.

cargo molecules, which provides a point of distinction between lysosomal degradation and sorting into exosomes (Figure 2a).[45,46] The ESCRT machinery triggers the formation of vesicles in late-endosomal MVBs,[47–50] whereas ceramide-enriched microdomains induce exosome budding through the lateral segregation of cargo within the late-endosomal membrane, protecting them from transport to lysosomes.[51] Once the late-endosomal cargo is sorted, the constituent exosomes are released to the extracellular environment upon fusion of the limiting membrane of MVBs with the plasma membrane.[9,52] The mechanisms that regulate the fusion of MVBs with the plasma membrane are unclear; however, Rab GTPases, such as Rab5 and Rab7, have been shown to regulate endocytic trafficking downstream of MVB biogenesis and cargo sequestration, whereas Rab27a, Rab27b and Rab35 control the secretory pathway and label MVBs for subsequent fusion with the plasma membrane, resulting in the release of exosomes.[45,53–55]

Microvesicles are produced through membrane-budding processes,[22] which follow an ordered pattern: the cellular trafficking of biomolecules towards the cell surface results in membrane protrusion, budding and, finally, the detachment of spherical bodies from specific regions of the plasma membrane enriched in lipid rafts (Figure 2b).[56] The formation of microvesicles might share some common features with exosome biogenesis. For instance, the ESCRT component TSG101, which is involved in the exosomal pathway, is known to interact with arrestin domain-containing protein 1 during microvesicle shedding.[57] Another common feature of both types of extracellular vesicle is that their formation is linked to regions of the plasma membrane that are enriched in ceramide, cholesterol and lipid rafts. During microvesicle budding, ceramide promotes membrane curvature,[58] a process that might resemble abscission steps in cytokinesis and could use actin-based motors.[59] Furthermore, the small GTP-binding protein ARF6, well known for its role in cell

Table 1 | Summary of extracellular vesicle isolation techniques

Method	Principle	Advantages	Disadvantages	References
Differential centrifugation	Sedimentation based on size and density	Gold standard Most widely used Suitable for large-volume isolations	Requires expensive ultracentrifuge Time consuming Recovery based on sedimentation efficiency No absolute separation of vesicle subpopulations Risk of contamination and formation of protein aggregates	Alvarez *et al.* (2012)[69] Johnstone *et al.* (1987)[164]
Density gradient centrifugation	Flotation based on density	Increases the purity by removal of contaminating protein aggregates	Requires expensive ultracentrifuge Time consuming Sucrose toxicity might limit downstream functional studies No absolute separation of vesicle subpopulations owing to overlapping density	Tauro *et al.* (2012)[72] Théry *et al.* (2006)[165]
Ultrafiltration	Separation based on size	Easy and fast	Small sample volume limitations Protein contamination Loss of yield owing to trapping in filter pores	Cheruvanky *et al.* (2007)[78] Merchant *et al.* (2010)[79]
Chromatography	Separation based on size	Increases purity and integrity Suitable for isolation from complex biofluids	Requires specialized equipment Small sample volume limitations Time consuming	Chen *et al.* (2011)[80] Lai *et al.* (2012)[81] Taylor *et al.* (1983)[82]
Affinity isolation	Separation based on affinity interactions	Increases purity High specificity to isolate subpopulations	Requires prior knowledge of vesicle characteristics Requires specific antibody Not suitable for large sample volumes Captured vesicles might not retain functionality after elution	Chen *et al.* (2010)[84] Clayton *et al.* (2001)[83]
Polymeric precipitation	Separation based on PEG precipitation	Quick and relatively cheap High yield	Low purity caused by contamination Low specificity	Burns *et al.* (2014)[166] Rekker *et al.* (2014)[71]
Microfluidic devices	Separation based on mechanics of fluid flow	Increases throughput and allows multiplexing Reduced cost, sample size and processing time	Not applicable to large sample volumes	Chen *et al.* (2010)[84]

Abbreviation: PEG, polyethylene glycol.

invasion and actin remodelling,[60,61] is thought to regulate the release of protease-loaded vesicles derived from the plasma membrane.[62] Crosstalk between ARF6 and Rho signalling pathways has been implicated in the release of microvesicles,[63] and commonalities have been observed between mechanisms governing microvesicle formation and membrane blebbing at the cell surface that increases cell motility by amoeboid projection.[64,65] Altogether, these observations indicate that actin motors and elements of the cytoskeleton might be involved in the formation of extracellular vesicles. Consistent with this, Nawaz *et al.*[11] have speculated that extracellular vesicle budding might occur as a result of cell extrusion and membrane blebbing at lipid rafts through 'spindle rocking' (previously observed in aberrant cytokinesis[66]).

Apoptotic bodies

Tumour cells undergoing apoptotic disaggregation can produce apoptotic bodies or apoptosomes[6,67]—relatively large membrane blebs formed by indiscriminate blebbing of the plasma membrane (Figure 2c). Apoptotic bodies contain fragmented nuclei as well as fragmented cytoplasmic organelles, which might be taken up by cells in the tumour microenvironment, thereby influencing the cellular response by transferring their oncogenic contents to recipient cells.[68]

Analysis of extracellular vesicles

Prior to the biochemical or high-throughput analyses of extracellular vesicles, their rapid and efficient isolation is required. A variety of isolation technologies exist (and more are being developed), and each technique provides unique advantages and disadvantages depending on the sample source, and the desired yield and purity.

Isolation of extracellular vesicles

Extracellular vesicles can be isolated by many different methods, including differential centrifugation, density gradient centrifugation, ultrafiltration, chromatography, affinity isolation, polymeric precipitation and by the use of microfluidic devices (Table 1). However, no 'one size fits all' approach exists, and all available methods have advantages and disadvantages. The method of choice should take into account the sample volume (for example, whether the sample is derived from biofluids or from cell-culture media), the purity, integrity and yield of extracellular vesicles required for specific downstream analysis (such as proteomic analysis or RNA profiling), as well as the available instrumentation and processing time. A comparison of isolation methods for different samples, such as blood plasma, milk, urine and cancer cell culture media, has been previously undertaken.[69–74] In addition to the existing, validated methods, novel and developing technologies are available for extracellular vesicle isolation.

Centrifugation

Currently, differential centrifugation, with or without size filtration, is the most widely used isolation method. This approach comprises three centrifugation steps: low speed (300–500 *g*, 5–10 min) to eliminate cells and cell debris; medium speed (10,000–20,000 *g*, 10–20 min) to

eliminate larger vesicles; and high speed (100,000*g*, 1–3 h) to pellet extracellular vesicles. Important considerations are the rotor type and the viscosity of the sample, which strongly influence the sedimentation efficiency and resolution of extracellular vesicle preparations.[75–77] To increase the purity of the yield, density gradient centrifugation using sucrose or iodixanol (OptiPrep™, AXIS–SHIELD, Norway) gradients can be applied as an additional clean-up step, to separate vesicles with different densities.

Ultrafiltration

On the basis of passage through filters with nanopores or micropores, extracellular vesicles can also be isolated by ultrafiltration, which does not require expensive equipment.[78,79] Although ultrafiltration is rapid, abundant protein contamination and retention of extracellular vesicles in membrane pores are among the factors that decrease extracellular vesicle yield.

Chromatography

Similarly to ultrafiltration, chromatography is used to isolate extracellular vesicles based on the concept that size differences alter their chromatographic retention times.[80–82] Although chromatographic isolation increases the purity of the yield, it does not reduce the processing time, and requires specialized equipment.

Affinity isolation

Immunoaffinity isolation can also increase the purity of extracellular vesicles, and enables the selective capture of specific subpopulations. Antibodies against proteins located on the surface of extracellular vesicles are precoated on beads or plates to either capture or deplete specific subpopulations of extracellular vesicles, which are isolated by high-affinity interactions with the desired antibody, and further separated by low-speed centrifugation or magnetic-bead techniques.[83] Dynabeads®-related products for the isolation of extracellular vesicle subpopulations have been developed by Life Technologies (UK). Chen *et al.*[84] have developed a microfluidic device that contains antibody-coated magnetic beads bound to a magnetic sensor on the surface of the chip, enabling the single-step capture of extracellular vesicles. Balaj *et al.*[85] have also reported a novel affinity isolation method using heparin-coated beads, but the application and validation of this method has been limited to date.

Polymeric precipitation

On the basis of polyethyleneglycol (PEG) precipitation of extracellular vesicles, System Biosciences (USA) have released a commercial isolation kit (ExoQuick™) that reduces hands-on time and yields high levels of extracellular vesicles, although the purity is lower than with some other isolation methods.

Qualitative and quantitative analysis

The characterization, determination of purity and quantification of isolated extracellular vesicles can be performed using various methods, some of which have been used for many years, whereas others are relatively novel (Table 2). A combination of different methods is often required to overcome some of the challenges related to extracellular vesicle detection, such as their small size and lack of distinct markers. Here, we provide a brief overview of qualitative and quantitative approaches for the analysis of extracellular vesicles. However, for a more comprehensive characterization of extracellular vesicles, additional methods could be used to determine their protein, RNA and lipid content.[7,86,87]

Electron microscopy techniques are commonly used to visualize extracellular vesicles. The vesicles can be mounted on grids, fixed, stained with a contrast dye and visualized by transmission electron microscopy (TEM).[88,89] Furthermore, specific proteins can be visualized using immuno-electron microscopy with gold-labelled antibodies, enabling the detection of subpopulations of extracellular vesicles. However, the dehydration and fixation treatments used for conventional TEM might alter the morphology of the vesicles, and certain subpopulations might not adhere to the grid. Cryo-electron microscopy might, therefore, be more suitable for studying the morphology of extracellular vesicles,[89] as it does not require any fixation or staining, enabling biological specimens to be preserved and visualized to near-atomic resolution. However, the application of cryo-electron microscopy requires highly sophisticated equipment and technical expertise. Other methods to examine the morphology and 3D structure of extracellular vesicles include scanning electron microscopy and atomic force microscopy.[90,91]

New methods based on light scattering, such as dynamic light scattering and nanoparticle tracking analysis, have emerged for the detection of single exosomes.[92–94] Another instrument that can be used for nanoparticle sizing, enumeration and charge measurement is qNano™ (Izon, New Zealand), which provides a label-free method for detecting charged particles passing through a nanopore via electrophoresis.

The application of flow cytometry to extracellular vesicle characterization has been limited by the existence of some subpopulations that are below the detection range of a conventional cytometer—for which vesicles must be >500 nm—but this limitation can be circumvented by attaching extracellular vesicles to antibody-coated beads, which can easily be detected by conventional flow cytometers.[83] This method does, however, preclude the analysis of single extracellular vesicles, as multiple vesicles attach to each bead. Interestingly, a new, fluorescence-based, high-resolution, flow cytometry method is able to detect different subsets of extracellular vesicles and to determine the phenotypes of single entities.[95]

Surface and/or intravesicular proteins can be analysed using a range of methods, including Western blot, ELISA and extracellular vesicle arrays.[96,97] Other sensitive qualitative and quantitative technologies have been developed to detect extracellular vesicles, such as magnetic-labelled (micronuclear magnetic resonance [μNMR] system) and nonlabelled (nanoplasmonic exosome [nPLEX]) methods, as well as a photosensitizer-bead detection system (ExoScreen).[98–100]

Table 2 | Summary of techniques for extracellular vesicle detection and characterization

Methods	Size detection range/ detection limit	Size distribution*	Concentration*	Marker detection*	References
Quantitative methods					
DLS	1 nm to 6 μm	+	–	–	Dragovic *et al.* (2011)[92] Gardiner *et al.* (2013)[93] Sokolova *et al.* (2011)[94]
qNano	70 nm to 10 μm	+	+	–	Momen-Heravi *et al.* (2012)[76] Momen-Heravi *et al.* (2012)[77]
Qualitative methods					
Western blot and ELISA	NA	–	–	+	Logozzi *et al.* (2009)[97] Raposo *et al.* (1996)[167]
Extracellular vesicle array	NA	–	–	+	Jorgensen *et al.* (2013)[96]
TEM	<1 nm	+	–	+	Raposo *et al.* (1996)[167] Yuana *et al.* (2013)[89]
SEM	~1 nm	+	–	+	Sharma *et al.* (2011)[90]
AFM	<1 nm	+	–	–	Sharma *et al.* (2011)[90] Sharma *et al.* (2010)[91]
Quantitative and qualitative methods					
NTA	50 nm to 1 μm	+	+	+	Dragovic *et al.* (2011)[92] Gardiner *et al.* (2013)[93]
Conventional flow cytometry	≥300 nm <300 nm (binding with beads)	– –	+ –	+ +	Clayton *et al.* (2001)[83]
Fluorescence high-resolution flow cytometry	~100 nm	–	+	+	Nolte-'t Hoen *et al.* (2012)[95] Nolte-'t Hoen *et al.* (2013)[168]
μNMR system	50–150 nm	–	+	+	Shao *et al.* (2012)[99]
nPLEX assay	NA	–	+	+	Im *et al.* (2014)[98]
ExoScreen	NA	–	+	+	Yoshioka *et al.* (2014)[100]

*+ indicates variable can be measured, – indicates it cannot. Abbreviations: AFM, atomic force microscopy; DLS, dynamic light scattering; μNMR, micronuclear magnetic resonance; NTA, nanoparticle tracking analysis; nPLEX, nanoplasmonic exosome; SEM, scanning electron microscopy; TEM, transmission electron microscopy.

Urogenital cancers

Cancers of the prostate, kidney and bladder are classified as urogenital cancers. Despite intensive research, no biomarkers are yet available to deliver accurate early detection or precise prognoses. Extracellular vesicles provide a novel target to potentially improve on these shortcomings (Figure 3).

Prostate cancer

Prostate cancer is the most commonly occurring cancer in men, but fewer than 20% of those affected will present with lethal disease.[1,2] Although the diagnostic regimen of digital rectal examination (DRE) combined with serum PSA screening has considerably improved the early detection of prostate cancers, it has also led to a dramatic increase in overtreatment of the disease. A major clinical challenge is to accurately distinguish patients with indolent disease from those with aggressive tumours that require increased intervention. Furthermore, patients who are under active surveillance would benefit from less-invasive monitoring assays to overcome the potential dangers associated with repeated needle biopsies.

PSA testing is approved by the FDA for diagnosis of prostate cancer, but has a considerable number of limitations: it shows a lack of sensitivity and specificity,[101] its levels in serum are elevated in some nonmalignant conditions and it does not provide a reliable indication about the metastatic potential of prostate cancer cells.[102] The FDA has approved a test in urine for the noncoding RNA of prostate cancer antigen 3 (*PCA3*) as a biomarker for prostate cancer—its upregulation is believed to be an exclusive characteristic of prostate cancer, and it shows improved specificity and sensitivity compared to PSA.[103,104] Early prostate cancer antigen (EPCA), an integral nuclear membrane protein unique to the nuclei of prostate cancer cells,[105,106] has been proposed as a potential serum biomarker, and protein kinase C α (PKCα)[107] as a tissue biomarker for early detection, but these proteins have not, as yet, undergone systematic validation.

Thus, although the application of next-generation genomic and proteomic technologies to blood, urine and prostate proximal fluids is likely to add new candidates to the already long list of putative RNA, DNA and protein biomarkers identified over decades of research (such as prostatic acid phosphatase, EPCA, glutathione-*S*-transferase π and α-methylacyl-CoA racemase), most of these require further validation, and they often lack sensitivity and specificity. Alternative strategies for biomarker discovery are, therefore, required, and extracellular vesicles can be considered a promising source for future investigations. They have gained considerable attention as candidate biomarkers to distinguish between

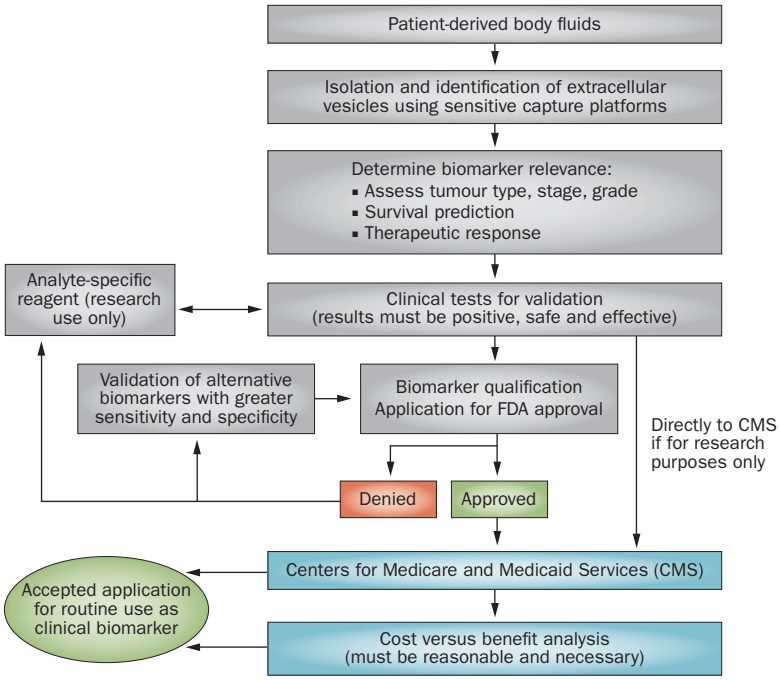

Figure 3 | Multistep validation of biomarkers from extracellular vesicles. A potential flowchart for the validation and clinical implementation of biomarkers based on extracellular vesicles. The flowchart shows a step-by-step process by which the profiling and discovery of exosomal cargo molecules could ultimately be translated into a clinically applicable biomarker signature. At each step defined goals and criteria must be met in order to proceed to the next level.[163]

indolent and aggressive forms of prostate cancer, and multiple studies have documented the presence of prostate-derived extracellular vesicles in body fluids as well as in prostate cancer cell lines and tissue.[102,108–117]

Plasma

Extracellular vesicles isolated from plasma harbour proteins specific to prostate cancer, such as phosphatase and tensin homolog (PTEN) and survivin. PTEN has been detected in patients with prostate cancer but not in plasma of healthy subjects, and survivin has been shown to be present at higher levels in the plasma of patients with prostate cancer compared with patients with BPH, or healthy individuals.[118,119]

Plasma extracellular vesicles have also been shown to contain a rich repertoire of RNA species. In the context of urological cancers, extracellular vesicles containing microRNA (miRNA) have attracted the most interest.[120] Interestingly, higher levels of miRNA were observed in plasma vesicles compared with vesicle-depleted supernatant,[121] suggesting either increased stability or selective packaging of miRNAs into extracellular vesicles. Bryant and colleagues[122] reported the differential expression of 12 miRNAs associated with extracellular vesicles in the serum and plasma of patients with prostate cancer (Table 3). miR-141 and miR-375 were among 11 miRNAs that were found at significantly higher levels in extracellular vesicles obtained from the serum of patients with metastatic, compared with nonmetastatic, prostate cancer.[122] Elevated levels of miR-141 in plasma or serum have been considered to distinguish patients with prostate cancer from healthy subjects,[33] and its expression has been associated with an increased Gleason score and advanced pathologic stages.[123]

A highly specific and sensitive method known as the proximity ligation assay has been used to quantify levels of prostasomes—extracellular vesicles secreted by epithelial cells in the prostate gland—in the plasma of patients.[114] This assay could offer a promising tool for the diagnosis and prognosis of prostate cancer.

Prostate tissues

Prostasomes are considered to provide the most accurate source of proteomic or transcriptomic biomarkers for prostate cancer. So far, however, only one study has reported the isolation of extracellular vesicles from metastatic prostate cancer tissue, documenting an altered expression of annexins A1, A3, A5 and dimethylarginine dimethylaminohydrolase 1.[124]

Prostate cancer cell lines

A large number of proteins have been found to associate with extracellular vesicles derived from prostate cancer cell lines (Table 3).[110,125–127] However, with the exception of fatty acid synthase, which has also been identified in extracellular vesicles derived from expressed prostatic secretions (EPS) in urine,[29] the majority of these proteins have not been identified in vesicles derived from urine, serum or plasma, which limits the use of prostate cancer cell lines in the identification of potentially useful biomarkers, as they fail to accurately recapitulate this disease.[26]

Prostate proximal fluids and urine

Prostate proximal fluids, such as seminal fluid and EPS, contain considerable amounts of extracellular vesicles and hence could represent ideal fluids for the discovery of extracellular-vesicle-derived biomarkers. To date, however, few systematic biomarker discovery studies have been conducted using these fluids.[26,28,29,128] At present, the lack of a systematic, high-quality biobanking system for prostate proximal fluids is a limiting factor, but this is expected to change in the future, following the approval of the *PCA3* diagnostic test in 2012, which uses post-DRE urine that contains EPS.

An in-depth proteomic study carried out in 2013 identified 877 exosomal proteins derived from EPS in urine, 14 of which were most-readily detectable in extracellular vesicles (Table 3).[29] Furthermore, urine-derived extracellular vesicles comprise a rich source of *N*-glycoproteins, as they originate from the endocytic pathway.[26] In a comprehensive study, >25 key *N*-linked glycan species were found to be associated with extracellular vesicles derived from EPS in urine, with levels that varied according to disease status.[129]

Other studies have also investigated the proteomic cargo of prostate-derived extracellular vesicles from urine.[130–133] Increased levels of α1-integrin and β1-integrin were found in urine exosomes of patients with metastatic prostate cancer, compared with patients

Table 3 | Candidate biomarkers for prostate cancer derived from extracellular vesicles

Markers	Source	Methodologies	Reference
PTEN	Plasma	Ultracentrifugation, Western blot, immunofluorescence	Gabriel *et al.* (2013)[118]
Survivin	Plasma	Ultracentrifugation, Western blot, ELISA	Khan *et al.* (2012)[119]
miR-107, miR-130b, miR-181a-2, miR141, miR-301a, miR-326, miR-331-3p, miR-375, miR-432, miR-574-3p, miR-22110, miR-625	Plasma, serum and urine	ExoMiR extraction, filtration, qRT-PCR	Bryant *et al.* (2012)[122]
ANXA1, ANXA3, ANXA5, DDAH1	Tissue	Ultracentrifugation, gel filtration chromatography, 2D–PAGE, mass spectrometry (MALDI–TOF)	Ronquist *et al.* (2010)[124]
CDCP1, CD151, CD147	PCCL	Ultracentrifugation, mass spectrometry (LC–MS/MS), bead immuno-isolation, Western blot	Sandvig *et al.* (2012)[127]
ANXA2, CLSTN1, FASN, FLNC, FOLH1, GDF15	EPS-urine PCCL	Ultracentrifugation, mass spectrometry (LC–MS/MS) Centrifugation, sucrose gradient, mass spectrometry (LC–Q–TOF)	Principe *et al.* (2013)[29] Utleg *et al.* (2003)[116]
PDCD6IP, XPO-1, ENO1	PCCL	Ultracentrifugation, mass spectrometry (LC–MS/MS)	Duijvesz *et al.* (2013)[125]
ACPP, LTF, DDP4, TGM4, MME, PSA, SEMG1, AZGP1, ANPEP, G3BP, PSMA, TMPRSS2, FASN, LGALS3, PSCA, KLK2, KLK11, TIMP1	EPS-urine	Ultracentrifugation, mass spectrometry (LC–MS/MS)	Principe *et al.* (2013)[29]
ITGA3, ITGB1	Urine	Ultracentrifugation, mass spectrometry (LC–MS/MS), Western blot, flow cytometry	Bijnsdorp *et al.* (2013)[130]
δ-catenin	Urine	Ultracentrifugation, immunoprecipitation, Western blot, electron microscopy	Lu *et al.* (2009)[131]
N-linked glycans	EPS-urine	Ultracentrifugation, mass spectrometry (MALDI–TOF)	Nyalwidhe *et al.* (2013)[129]
TMPRSS2–ERG, *PCA3* transcripts	Urine EPS-urine	Ultracentrifugation, filtration, sucrose gradient, PCR Centrifugation, filtration, RT-PCR, gene expression	Khan *et al.* (2012)[119] Dijkstra *et al.* (2014)[134]

Abbreviations: 2D-PAGE, 2D polyacrylamide gel electrophoresis; ACPP, prostatic acid phosphatase; ANPEP, aminopeptidase N; ANX, annexin; AZGP1, zinc-alpha-2-glycoprotein; CDCP, CUB domain-containing protein 1; CLSTN1, calsyntenin-1; DDAH1, dimethylarginine dimethylaminohydrolase 1; DDP4, dipeptidyl peptidase-4; ENO1, enolase-1; EPS, expressed prostatic secretions; FASN, fatty acid synthase; FLNC, filamin-C; FOLH1, folate hydrolyase 1; G3BP, galectin 3 binding protein; GDF15, growth/differentiation factor 15; ITG, integrin; KLK, kallikrein; LC–MS/MS, liquid chromatography–mass spectrometry; LC–Q–TOF, liquid chromatography, quadrupole, time-of-flight; LGALS3, galectin-3; LTF, lactotransferrin; MALDI–TOF, matrix-assisted laser desorption/ionization–time-of-flight mass spectrometry; miR, microRNA; MME, neprilysin; PCA3, prostate cancer antigen 3; PCCL, prostate cancer cell line; PDCD6IP, programmed cell death 6-interacting protein; PSA, prostate-specific antigen; PSCA, prostate stem cell antigen; PSMA, prostate-specific membrane antigen; PTEN, phosphate and tensin homolog; qRT-PCR, quantitative reverse transcription PCR; SEMG1, semenogelin-1; TGM4, protein-glutamine gamma-glutamyltransferase 4; TIMP1, tissue inhibitor of matrix metalloproteinase 1; TMPRSS2, transmembrane protease serine 2; XPO-1, exportin-1.

with nonmetastatic disease or those with BPH.[130] Likewise, δ-catenin associated with extracellular vesicles has been found at high levels in urine,[131] whereas prostate-specific membrane antigen and prostate stem cell antigen have also been identified in urine-derived exosomes of patients with prostate cancer.[26,29,129] In addition to exosomal protein markers, some transcriptomic changes have also been identified, including those affecting the expression of *TMPRSS2* and, of course, *PCA3*.[112,134] Urine and EPS-enhanced urine are suitable clinical samples for the isolation of extracellular vesicles owing to their ease of collection and considerable protein content.[129] Furthermore, the composition of urine is far less complex than that of blood, providing a sample type that is straightforward to analyse by current proteomic technologies. Further studies are needed to validate whether the content of extracellular vesicles derived from patients with prostate cancer provides a source, so far relatively unexplored, for the discovery of novel biomarkers.

Kidney cancer

Accounting for 3% of all human cancers, renal cell carcinoma (RCC) has a high frequency of relapse and a mortality rate that reaches >40%.[135] RCC subtypes include clear cell, papillary, chromophobe and collecting duct RCC—clear cell RCC (ccRCC) accounts for 75–80% of all renal tumours.[136] An accurate diagnosis of RCC subtype is critical, as the different subtypes have different biological features and clinical outcomes and, consequently, different prognoses and responses to therapy. In the absence of reliable biomarkers to facilitate early detection, diagnosis mainly relies on the renal histopathological profiles.

Most biomarkers so far identified are related to angiogenesis, which has a critical role in RCC development and progression. In ccRCC, hypermethylation or mutation of the von Hippel–Lindau (*VHL*) tumour suppressor gene frequently occurs (in 35–57% of cases), leading to altered regulation of the hypoxia-inducible factor (*HIF1* and *HIF2*) genes and consequent constitutive activation of angiogenesis.[137] Attempts to correlate the levels of expression of carbonic anhydrase IX (CAIX), β-catenin and HIF2α with the progression and severity of RCC have been made, but these proteins have not yet been fully confirmed as reliable biomarkers for the diagnosis of RCC. Studies comparing the miRNA expression profiles of normal and RCC tissue specimens have shown that miRNAs that target tumour suppressors are upregulated, whereas those that target oncogenes are downregulated in RCC, as has been reviewed previously.[138] The dysregulation of miRNAs has profound biological implications, as they target molecules implicated in RCC progression, such as PTEN, VHL, HIF, vascular endothelial growth factor (VEGF) and mammalian target of rapamycin (mTOR). These observations have prompted studies aimed at identifying miRNAs within the circulation and urine of patients with RCC, which have been reviewed elsewhere.[138] An analysis of

Table 4 | Candidate biomarkers for kidney cancer derived from extracellular vesicles

Markers	Source	Methodologies	Reference
VEGF, FGF, angiopoietin 1, ephrin-A3, MMP-2, MMP-9	Cancer stem cells	Ultracentrifugation, flow cytometry, immunohistochemistry	Grange *et al.* (2011)[14]
miR-200c, miR-92, miR-141, miR-19b, miR-29a, miR-29c, miR-650, miR-151	Cancer stem cells	Ultracentrifugation, microarray analysis, qRT-PCR	Grange *et al.* (2011)[14]
Fas ligand, Bcl2-L-4	RCC cells	Centrifugation, filtration, flow cytometry, Western blot, ELISA	Yang *et al.* (2013)[145]
Lysophosphatidylethanolamine metabolite	Urine	Ultracentrifugation, mass spectrometry (LC–MS/MS)	Del Boccio *et al.* (2012)[146]
MMP-9, ceruloplasmin, PODXL, DKK4, CAIX	Urine	Ultracentrifugation, Optiprep™ density gradient, mass spectrometry (LC–MS/MS), Western blot	Raimondo *et al.* (2013)[147]

Abbreviations: Bcl2-L-4, apoptosis regulator BAX; CAIX, carbonic anhydrase IX; DKK, Dickkopf-related protein; FGF, fibroblast growth factor; LC–MS/MS, liquid chromatography–mass spectrometry; miR, microRNA; MMP, matrix metalloproteinase; PODXL, podocalyxin; qRT-PCR, quantitative reverse transcription PCR; RCC, renal cell carcinoma; VEGF, vascular endothelial growth factor.

circulating serum miRNAs revealed that a small subset of 36 miRNAs were upregulated not only in the serum, but also in tissue samples of patients with RCC.[139] As miR-378 is overexpressed in RCC serum, it has been proposed as a potential biomarker,[140] but with conflicting results.[141] Another miRNA that is highly expressed in the serum of patients with RCC, the levels of which have been shown to decrease after surgery, is miR-210, which is directly upregulated by HIF.[142] The levels of miR-15a are upregulated in the urine of patients with ccRCC, but downregulated in patients with oncocytoma, suggesting that this biomarker shows tumour-subtype specificity.[143]

Extracellular vesicles in RCC

The pathogenic role of extracellular vesicles has been extensively investigated in several cancers, but few studies have addressed their role in RCC. Extracellular vesicles released from renal cancer stem cells were shown to influence the tumour microenvironment, thereby promoting angiogenesis, tumour invasion and premetastatic niche formation in the lungs,[14] whereas those from differentiated tumour cells did not display these properties. Comparative studies of RNA species from these two different extracellular vesicle sources revealed that those from cancer stem cells were enriched in mRNAs encoding proteins involved in angiogenesis, such as fibroblast growth factor, VEGF, ephrin-A3, angiopoietin 1 and matrix metalloproteinase (MMP)-2 and MMP-9; additionally, 24 miRNAs were significantly upregulated and 33 miRNAs were downregulated in extracellular vesicles from these cells compared with extracellular vesicles from differentiated tumour cells.[14] The targets of these miRNAs, as predicted by gene ontology analysis, have been implicated in the regulation of cell proliferation, transcription, nucleic-acid binding, expression of adhesion molecules and metabolic processes. In particular, extracellular vesicles derived from renal stem cells were enriched in miR-29c, miR-19b and miR-151, which are also overexpressed in RCC, miR-92, miR-141 and miR-200c, which are significantly upregulated in several cancers, and miR-29a, miR-151 and miR-650, which are associated with tumour invasion and metastases.[138,144] Extracellular vesicles derived from RCC have been proposed to favour immune evasion as they contain Fas ligand, and induce apoptosis of T cells by increasing the levels of caspases and apoptosis regulator BAX (Bcl2-L-4), and decreasing the levels of apoptosis regulator Bcl-2.[145]

Few studies have addressed the potential use of urinary extracellular vesicles as a diagnostic tool for RCC. A comparative analysis of urinary extracellular vesicles performed using a hyphenated microLC-Q-TOF-MS (capillary liquid chromatography, quadrupole, time-of-flight mass spectrometry) platform demonstrated a differential lipid composition between the exosomes of patients with RCC and those of healthy control subjects.[146] Proteomic analysis of urinary extracellular vesicles has facilitated the identification of an RCC-specific 'fingerprint', containing proteins such as MMP-9, podocalyxin (PODXL), Dickkopf-related protein 4 (DKK4), carbonic anhydrase IX (CAIX) and ceruloplasmin (Table 4).[147] These preliminary studies suggest that urinary extracellular vesicles could provide a tool for identifying new biomarkers for RCC, although further studies are needed.

Bladder cancer

Bladder cancer is one of the five most frequent malignancies in developed countries and, among genitourinary tract malignancies, is second only to prostate cancer.[1] Early diagnosis of non-muscle-invasive papillary tumours followed by early treatment significantly improves prognosis, whereas prognosis is less favourable when muscle invasion is present at the time of diagnosis. Invasive bladder carcinomas show elevated resistance to chemotherapy, and, therefore, have a high recurrence rate, with high patient mortality.[148] Constant surveillance of these patients is required, and is carried out by combining cystoscopy, an invasive and costly, but very sensitive technique, with urinary cytology, which is specific, but less sensitive. As early diagnosis and monitoring of bladder cancer is critical, the discovery of new, sensitive biomarkers is of paramount importance. Cytology can be improved by combination with either an immunofluorescence assay that detects mucins associated with exfoliated bladder cells,[149] or with *in situ* hybridization, which can detect aneuploidy in chromosomes 3, 7 and 17, and loss of the 9p21 locus of the tumour suppressor gene p16.[150]

Table 5 | Candidate biomarkers for bladder cancer derived from extracellular vesicles

Markers	Source	Methodologies	Reference
Resistin, GTPase NRas, MUC4, EPS8L1, EPS8L2, EHD4, G3BP, RAI3, GSA	Urine	Ultracentrifugation, in-gel digestion, mass spectrometry (LC–MS/MS)	Smalley *et al.* (2008)[30]
TACSTD2 (one of 107 candidates, 24 validated)	Urine	Ultracentrifugation, flow cytometry, mass spectrometry (LC–MS/MS, MRM-MS), ELISA	Chen *et al.* 2012)[25]
β1 and α6 integrins, CD36, CD44, CD73, CD10, MUC1, basigin, 5T4	BCC/urine	Ultracentrifugation, flow cytometry, in-gel digestion, mass spectrometry (MALDI–TOF/TOF)	Welton *et al.* (2010)[157]
EDIL-3	BCC/urine	Ultracentrifugation, sucrose/D_2O cushion, mass spectrometry (LC–MS/MS)	Beckhan *et al.* (2014)[158]
LASS2, GALNT1	Urine	Ultracentrifugation, NanoSight, microarray, PCR	Perez *et al.* (2014)[159]

Abbreviations: 5T4, trophoblast glycoprotein; D_2O, deuterium oxide; EDIL-3, epidermal growth factor (EGF)-like repeat and discoidin I-like domain-containing protein 3; EHD4, EH domain-containing protein 4; EPS8L, epidermal growth factor receptor kinase substrate 8-like protein; G3BP, galectin 3-binding protein; GALNT, *N*-acetylgalactosaminyltransferase; GSA, α subunit of GsGTP binding protein; LASS2, LAG1 longevity assurance homolog 2; MALDI–TOF, matrix-assisted laser desorption/ionization–time-of-flight mass spectrometry; MRM-MS, multiple reaction monitoring-mass spectrometry; MUC, mucin; RA13, retinoic acid 13; TACSTD2, tumour-associated calcium-signal transducer 2.

A number of urinary biomarkers have been investigated. Proteomic analysis has revealed that levels of the nuclear mitotic apparatus protein 1 (NUMA1) are significantly elevated in the urine of patients with bladder cancer compared with normal subjects.[151] Several studies have found that the immunoassay used to detect NUMA1 is more sensitive than cytologic evaluation, ranging from 60% to 90%, as previously reviewed.[152] However, the specificity is significantly lower, owing to a number of interfering conditions, such as haematuria, inflammation and infections, which might give rise to false-positive results.[153] Another extensively studied urinary biomarker is bladder tumour antigen (BTA, identified as human complement factor H-related protein), the test for which has been used in the screening of a population of patients with suspected bladder cancers, as well as in surveillance for recurrence, but which should not be used as absolute evidence for the presence of bladder cancer, as several conditions not related to tumours might generate false-positive results. Fragments of cytokeratins 8, 18 and 19 released into the urine and detected by ELISA have also been evaluated as biomarkers of bladder cancer, but the specificity and sensitivity of the results have been discordant, as reviewed elsewhere.[152] Elevated urinary levels of the antiapoptotic protein survivin and the nuclear transcription factors BLCA-1 and BLCA-4 have also been detected with high sensitivity and specificity in patients with bladder cancer. However, these assays all require a more precise definition of the cut-off values, and better standardization, before entering into clinical use.

Profiling of gene mutations could also provide diagnostic and/or prognostic information for bladder cancer. Mutation of *TP53* (which encodes p53) in bladder cancer cells has been associated with a highly aggressive phenotype,[154] whereas mutation of *FGFR3* (which encodes the fibroblast growth factor receptor 3) has been associated with a low tumour grade and low risk of recurrence.[155] Another promising marker is the gene encoding the mitosis regulator Aurora kinase A—a high degree of amplification of this gene has been associated with high tumour grade.[156]

Diagnostic potential of extracellular vesicles

In bladder cancer, extracellular vesicles released into urine from the bladder carry the signature of the tumour cells of origin, and can, therefore, be exploited as diagnostic factors. The proteomic profiles of extracellular vesicles in the urine of patients with bladder cancer have previously been characterized. Smalley *et al.*[30] identified several proteins that were present at elevated levels in extracellular vesicles that are potentially involved in tumour progression, including components of the epidermal growth factor (EGF) pathway, the α subunit of the G protein Gs, resistin and retinoic acid protein 3. Welton *et al.*[157] extended the proteomic studies of urinary extracellular vesicles with gene ontology analysis, as well as demonstrating a strong association of the identified proteome with exosomes derived from a bladder cancer cell line (Table 5). On the basis of proteomic analysis in extracellular vesicles, Chen *et al.*[25] proposed that tumour-associated calcium-signal transducer 2 (TACSTD2) has diagnostic value as a biomarker owing to its presence at high levels in patients with bladder cancer.

Tumour cells release extracellular vesicles that influence surrounding or distant cells, and that might, therefore, transfer cargo that can modify the phenotypes of recipient cells.[13] Extracellular vesicles derived from bladder cancer might promote cancer progression by delivering the protein EGF-like repeat and discoidin I-like domain-containing protein-3 (EDIL-3), an integrin ligand implicated in angiogenesis.[158] Extracellular vesicles have been shown to transfer genetic information between cells, as they carry functional RNA transcripts, implying that the extracellular vesicle transcriptome might contain markers for the cell of origin. In 2014, Perez *et al.*[159] investigated the potential application of the RNA content of urinary extracellular vesicles (Table 5) in the diagnosis of bladder cancer. Using microarray technology followed by PCR validation, they generated a list of differentially expressed genes in urinary extracellular vesicles from patients with cancer compared with those from cancer-free controls. *LASS2* and *GALNT1*, which encode proteins involved in cancer progression and metastasis, were found only in extracellular

vesicles from the urine of patients with bladder cancer, whereas *ARHGEF39* and *FOXO3*, which encode a guanine nucleotide exchange factor that promotes cell proliferation and a transcriptional activator involved in apoptosis, respectively, were only present in controls. Extracellular vesicles also contain miRNAs, which they protect from degrading enzymes, and several studies have addressed the potential diagnostic and/or prognostic values of miRNAs as markers for bladder cancer. In particular, the presence of miR-1224-3p, miR-135b and miR-15b, as well as the ratio of miR-126 to miR-152 in urinary pellets, has been shown to correlate with a positive bladder cancer diagnosis.[120] These data suggest that profiling miRNAs derived from extracellular vesicles from body fluids could result in the identification of promising new biomarkers for tumours of the bladder and, perhaps, other urological cancers.

Conclusions

Despite the considerable research efforts applied to cancer biomarker discovery, a deficit of reliable markers to facilitate early detection, accurate prognosis and reliable prediction of response to treatment still remains. The quest for clinically relevant biomarkers for urogenital cancers remains an unmet challenge. Ongoing efforts for the identification of biomarkers include a variety of profiling technologies that are aimed at the discovery of genetic/epigenetic, proteomic and lipidomic alterations. Although lipidomic analyses of extracellular vesicles are still relatively rare, we expect that the recently revived interest in cancer metabolism will result in an increase in such studies in the future.[160]

The identification of molecular signatures in biological fluids could create 'liquid biopsies', which would effectively overcome many of the challenges associated with traditional tissue sampling (such as invasiveness and tumour heterogeneity). In this regard, the analysis of extracellular vesicles derived from body fluids could offer an especially attractive source of biomarkers, since these vesicles are thought to reflect the molecular composition of the secreting cell. In urogenital cancers, increased levels of extracellular vesicles during tumorigenesis might serve as indicators for disease surveillance. Interestingly, molecular cargoes, such as nucleic acids and proteins, seem to be selectively sorted into extracellular vesicles, as recently shown for neural precursor cells exposed to proinflammatory cytokines,[161] and these regulated mechanisms in cancer cells can provide access to a tumour-specific repertoire. For instance, several proteomic studies have revealed the presence of urinary extracellular vesicles that contain candidate proteins unique to cancer types that include a broad range of urogenital diseases.[25,26,29,102,110,112,147,157,162] Such tumour-specific vesicles are easily captured from urine using established isolation procedures, which enables repeated tissue sampling.

One of the current challenges for the implementation of biomarkers based on extracellular vesicles in clinical practice is the development of isolation and detection methods that are compatible with current practices (Figure 3). The development of robust techniques and sensitive capture platforms that use readily accessible body fluids, particularly urine, could offer novel approaches for disease staging and diagnosis. Current efforts to systematically catalogue the nucleic acid, protein and lipid constituents of extracellular vesicles isolated from richly annotated clinical samples could ultimately help in developing sensitive and selective capture platforms directed towards specific extracellular vesicle subpopulations. Advances in next-generation sequencing and mass-spectrometry-based proteomics and metabolomics are likely to enable appropriate candidates to be established in the near future.

Review criteria

We searched for original articles in the PubMed database published between 1980 and 2014 containing the search terms "extracellular vesicles", "exosomes", "microvesicles", "microparticles", "ectosomes", "apoptotic bodies", "prostate cancer", "bladder cancer" and "kidney cancer", alone and in combination. All papers identified were English-language, full-text articles. Papers were prioritized on the basis of relevance and we apologize to authors of manuscripts that might have been missed.

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Author contributions

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