



The Role of Urinary Extracellular Vesicles in Kidney Cancer: Diagnostic and Therapeutic Potential

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Abstract

Renal cancer ranks as the 14th most common cancer globally, with renal cell carcinoma (RCC) being the primary variant, arising from renal tubular epithelial cells; clear cell RCC constitutes about 80% of cases. Despite their limitations, surgery and targeted therapy remain the mainstays of RCC treatment. Regardless of advancements in RCC research, substantial obstacles continue to exist, such as delayed diagnosis, advanced distant metastasis, and drug resistance. As urine is an easily accessible biofluid, the identification of EVs has paved the way for novel biomarker research. Urinary extracellular vesicles (uEVs) are a novel source of biomarkers with potential applications in cancer detection and management, utilizing a less invasive approach. New data indicate that uEVs are crucial in several areas of RCC, containing tumor development, metastasis, immune evasion, and response to drugs. These vesicles facilitate intercellular communication by transporting a variety of bioactive substances, including RNA, DNA, proteins, and lipids, and are released into the extracellular space by the majority of cell types. uEVs RNAs and proteins are presently being investigated for their possible application as diagnostic biomarkers for different types of kidney cancer. This review summarizes the most recent research examining the potential of uEVs cargo as a biomarker for the diagnosis, prognosis, and treatment of renal cancer.

Key words extracellular vesicles, urinary extracellular vesicles, renal cell cancer, urine, biomarker

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Introduction

Kidney malignancies rank as the ninth most prevalent cancer in men and the fourteenth in women globally. Even with progress in early detection and treatment, the incidence of kidney cancer is still increasing, especially in lower-income nations where healthcare systems are not adequately equipped to handle the escalating cancer challenge [1]. As reported by GLOBOCAN 2020, there were 431,288 new cases and 179,386 new fatalities associated with kidney cancer. The mortality and incidence rates of kidney tumor stand at 6.1 and 2.5 for males, while for females, they are 3.2 and 1.2, respectively [2]. Kidney malignancies are categorized according to their histological characteristics and require distinct targeted treatments. Consequently, new biomarkers for diagnosis and prognosis must be explored to improve the overall survival of kidney cancer.

Extracellular vesicles (EVs) are membrane-bound particles composed of a lipid bilayer that carries a variety of biological factors, including nucleic acids, proteins, metabolites, and lipids. EVs are available in different forms and can generally be divided into larger and smaller groups. They consist of exosomes, large oncosomes, apoptotic bodies, migrasomes, ectosomes (shedding microvesicles), and exosomes. Large extracellular vesicles are generally identified with diameters greater than 200 nm. This category includes different types like large oncosomes, apoptotic bodies, migrasomes, and ectosomes/shedding microvesicles (**Figure 1**). Conversely, small extracellular vesicles consist of vesicles that have diameters smaller than 200 nm. These consist of exosomes, exomeres, and a particular group of ectosomes/shedding microvesicles (**Figure 2**) [3-6]. Body fluids, such as blood, plasma, serum, and urine, along with a tissue medium for culture, serve as valuable sources of EVs [7]. A recent study indicated that EVs can be obtained from donor cells and transferred to recipient cells, thereby serving as a novel method of intercellular communication [8]. Moreover, EVs possess biomarkers that may be used to identify future metastatic sites. Therapeutic strategies may target EVs, inhibit specific organ uptake, target EV-induced alterations in possible metastatic sites, and utilize EVs as a drug delivery channel [9]. The prospective function of EVs in identifying and treating diseases with optimal accuracy has generated considerable research interest. This review summarizes the current research examining the potential utility of urinary extracellular vesicle (uEVs) cargo as biomarkers for prognosis, diagnosis, and medical therapy in kidney cancer.

Urinary extracellular vesicles

uEVs have garnered attention as a significant class of tumour biomarkers since their identification in 2004 [10]. EVs initially appear in human urine [11] and are commonly secreted from all nephron sections. Following their origin, renal tubular epithelial cells possess multivesicular structures at the apical surface, and urine exosomes contain apical membrane proteins from all cell types across the nephron [12, 13]. Thus, uEVs are appropriate foundational materials for identifying biomarkers applicable to many disease mechanisms, including cancer.

Physiological functions of uEVs

Growing evidence suggests that EVs excreted in urine can be internalized by other cells, hence influencing their function, indicating the existence of intra-nephron interaction along the urinary lumen [14]. Studies using electron microscopy have shown that cilia in vitro allow proximal tubular epithelial cells to internalize EVs [12]. Additionally, in vitro investigations have demonstrated that tubular cells may internalize EVs produced

from collecting ducts, thereby transmitting aquaporin 2 (AQP2) [15]. By inducing a profibrotic phenotype in cultured tubular epithelial cells, podocyte-derived EVs may have discovered a new method of glomerular-tubular communication [16, 17].

Furthermore, uEVs are likely to play a major role in elimination because they accumulate in the bladder and are subsequently expelled through urine, which is a heterogeneous mixture of uEVs. The major route of EVs elimination, including circulating EVs, is not yet known to be excreted through urine; rather, it is unclear whether EVs from the urinary tract are expelled from urine. Research on the physiological roles of uEVs is still in its early stages of development.

uEVs isolation methods

Innovation in the scientific and technological fields has driven the advancement of various EV isolation techniques. However, there has yet to be an ideal approach. Consequently, when designing an EV study, it is imperative to select the isolation method according to the intended downstream application (protein or nucleic acid isolation, biomarker finding, or functional assays) and the biological fluid from which the EVs will be collected (cultured cell media, urine, serum, plasma) [18-20]. The isolation of uEVs requires consideration of numerous practical factors [13, 21]. In research using urine extracellular vesicles, upholding ideal storage conditions for urine samples to avert proteolysis is crucial. Storage at 80°C, as opposed to at 4 °C or 20°C, is advantageous for avoiding degradation. However, the use of freshly processed urine is the most effective approach [22].

uEVs have been isolated using various methods, including ultracentrifugation, chemical precipitation, size-exclusion chromatography, and ultrafiltration technology [23]. Each technique utilises specific biophysical or biochemical characteristics of uEVs, including size, mass weight, structure, charge, and surface proteins, to facilitate their isolation. The two most common types of isolation procedures are ultracentrifugation (UC) and density-gradient ultracentrifugation (dUC) [24]. However, under pathological conditions, the resultant uEVs pellet is contaminated with prevalent urine proteins, including Tamm-Horsfall protein (THP, or uromodulin) and albumin. THP is derived from a glycosylphosphatidylinositol-linked protein located in the apical membrane of the thick ascending limb of the loop of Henle and is released into the urine through proteolytic processing [25]. THP generate extensive fiber channels that can restrict uEVs in urine and affect filtration mechanisms [26]. Therefore, methods to reduce or eradicate THP in the urine before uEVs isolation are necessary to improve the final production. Advanced ultracentrifugation is the predominant technique employed [27].

Table 1 outlines the various techniques for isolating uEVs.

Characterization methods

The widespread interest and complexity of uEVs have led to the invention and application of numerous approaches for their characterization. The currently used protocols and commercial kits that assert the isolation or purification of exosomes or EVs to a high standard are not capable of completely separating EVs from non-EV entities [38], making EV characterization a challenging task. Non-EV entities, for example, the argonaut 2 protein complex and lipoproteins, also contain components found in EVs [39, 40]. Furthermore, the results can be difficult to interpret because of the variety of methods and the composition of recovered EVs, which are influenced by factors such as experimental system variability, investigator expertise, and the apparatus employed. To ensure that biomarkers are linked to EVs and are not contaminated, EV detection and characterization should be evaluated using a variety

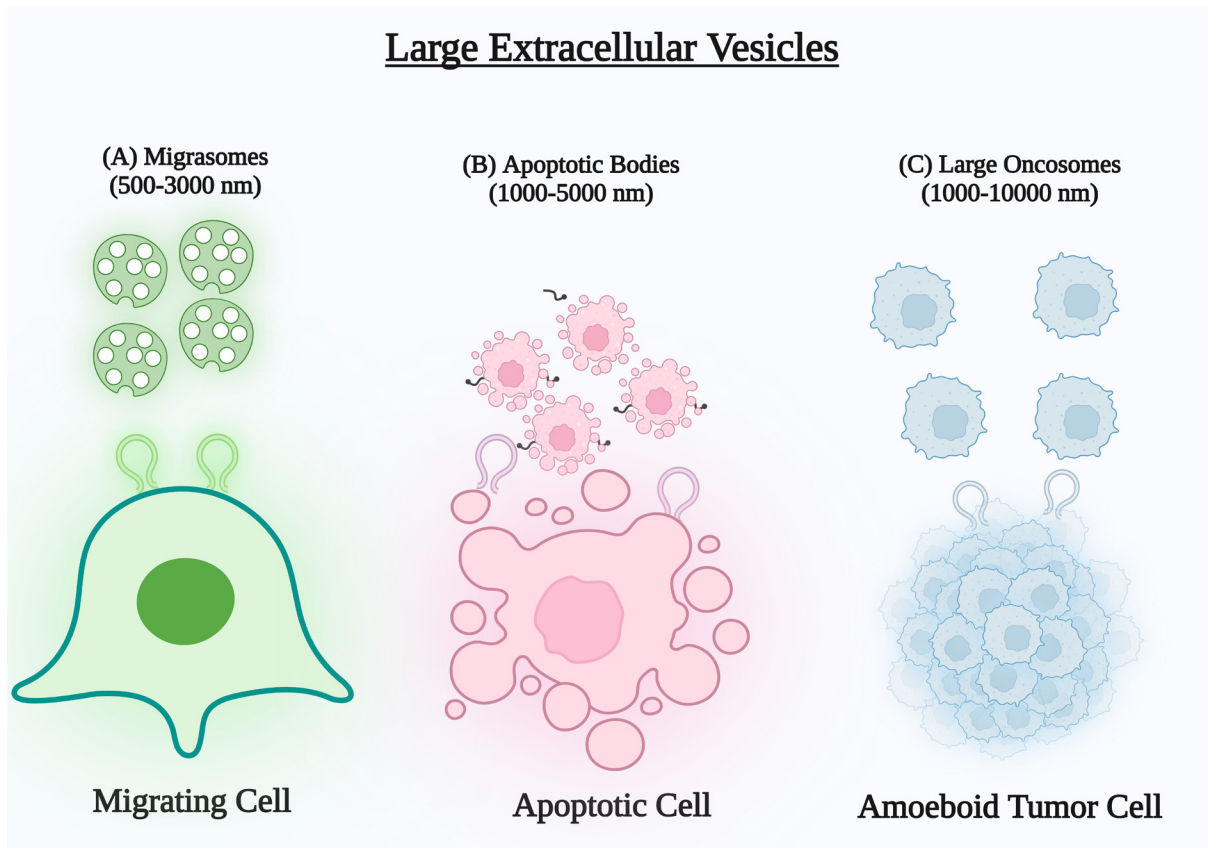


Figure 1 (A) Migrasomes are produced during cell migration at the ends or intersections of retraction fibres. They are then released into the extracellular matrix when the fibres separate. (B) Apoptotic bodies are discharged from the cell by membrane projections or blebs formed during apoptosis and released into the extracellular matrix. (C) Oncosomes are frequently produced from protrusions on the outer layer of amoeboid tumour cells and have been discovered to convey cancerous substances.

of complementary approaches, as per the MISEV2018 guidelines [18].

Unfortunately, no single technique can accurately describe the morphology, size, amount, and content of uEVs. Modern methods employed for the morphological characterization of EVs encompass transmission electron microscopy (TEM), cryogenic electron microscopy (cryo-EM), atomic force microscopy (AFM), and super-resolution fluorescence microscopy, with TEM being the most prevalent [41]. These methods are not always compatible with one another or can provide images of equal quality, because they provide distinct details regarding the structure and size distribution of uEVs. The techniques employed to quantify the size distribution and count of uEVs include nanoparticle tracking analysis (NTA) and tunable resistive pulse sensing (TRPS) [42, 43]. Based on Brownian motion, NTA offers the concentration and size distribution of uEVs particles within a certain detection range; however, it cannot rule out non-EV entities. Therefore, the particle count obtained from NTA may have been inflated. TRPS is a more precise way for measuring the particle size, amount, and surface charge [44, 45]. The concentration and size distribution of uEVs in a sample can be estimated by evaluating a large number of trajectories. Nevertheless, this technique is limited by the brief measured trajectories of in-focus and out-of-focus vesicles and particles, which may result in an inaccurate estimation of the particle concentration [46].

Finally, to enhance the integrity of uEVs preparation, co-isolated

components must be analyzed (e.g., THP in urine) [18]. Although immunoblotting, TEM and NTA are the most common methods for characterizing EVs [47], there is a vast array of approaches that are currently being developed and will contribute to better EV characterization in the future. One way to achieve good EV characterization is to combine some of the strategies that have been mentioned [48].

EVs in renal carcinoma

Kidney cancer is the fifth most commonly diagnosed form of cancer among males in Europe [49]. It is among the three most prevalent urological malignancies, following bladder and prostate cancer. RCC is the predominant form of kidney malignancy and ranks among the most prevalent urologic malignancies, accounting for nearly 90% of all renal malignancies [50]. RCC originates in the renal tubules, which purify the blood and generate urine. Furthermore, RCC commonly metastasizes to other organs in advanced stages, such as the bones, lungs, or brain [51]. Pathologically, the predominant subtypes of RCC are clear cell (75%–85%), papillary (10%–15%), and chromophobe (5%–10%). Among these common subtypes, clear cell renal cell carcinoma (ccRCC) has the lowest survival rate [52]. The prevalent metastatic locations of ccRCC are the lungs (54%), bones (18%), lymph nodes (16%), and the liver (6%) [53].

The diagnosis of RCC is frequently incidental, as many cases

Table 1. Different methods for isolating uEVs with pros and cons of each method.

Techniques	Methods of Isolations	Pros	Cons	Ref
Ultracentrifugation	(1) Progressive ultracentrifugation		(1) Processing a single sample requires 5-7 hours, is susceptible to contamination by highly abundant proteins, and requires costly machinery.	[28-30]
	(2) Double-cushion ultracentrifugation	(1) Results are reproducible, demonstrating many intact proteins and nucleic acids.		
	(3) Sucrose gradient ultracentrifugation	(2) Reduced contamination of highly abundant proteins.	(2) Extended processing duration; challenging separation methods; costly apparatus.	
	(4) Ultracentrifugation size exclusion chromatography			
Filtration	(1) Nanomembrane filtration	Reduced processing duration (0.5-2 hours); several samples can be simultaneously processed; cost-effective; applicable in clinical environments.	Risk of membrane blockage, specimen loss, and contamination from abundant proteins.	[31, 32]
	(2) Micromembrane filtration			
Precipitation	Precipitation by ExoQuick-TC	Reduced processing time (0.5–2 hours); cost-effective; yields RNA in its intact form; applicable to therapeutic settings.	Protein purity is low; the protocol has been modified.	[33]
Hydrostatic dialysis	Hydrostatic filtration dialysis	Appropriate for any downstream analysis; low cost, basic system; effective preparation and concentration for biobanking reasons.	Low protein purity compared to ultracentrifugation, but acceptable; THP contamination present. Compared to ultracentrifugation, large vesicles (>500 nm) were minimal, small EVs (60-140 nm) were rare, and EV-like particles (<40 nm) were more prevalent.	[34, 35]
Acoustic trapping	Polystyrene beads model	Quick, automated, suitable with low volumes, and resilient; does not affect trapped vesicle integrity or miRNA content.	Device parallelization may not be possible if an amplifier drives the piezo.	[36, 37]

remain asymptomatic until advanced stages of the disease. Consequently, the diagnosis of RCC is frequently postponed until the disease has progressed, with 30% of patients presenting with metastasis at diagnosis and an additional 30% developing metastasis during the disease's progression [54]. The gold standard solution for the management of localized kidney cancer is kidney surgery. Such procedures include radical nephrectomy, which involves the removal of the entire kidney, and partial nephrectomy, which involves the removal of only the section of the kidney that is malignant [55]. Additional methods for treating kidney tumor include radiation therapy, chemotherapy, targeted therapies, cryoablation, radiofrequency ablation, and microwave ablation [56, 57]. Furthermore, engineered EVs are promising carriers for RCC to enhance our understanding of targeted therapies.

Multiple ground-breaking investigations have demonstrated the potential of EVs in RCC diagnosis. CA9, CD70, and CD147, notable markers expressed in ccRCC tumour tissues, are also found in secreted EVs. These proteins can be used as reliable

biomarkers for tumor-specific, noninvasive detection approaches since their expression in EVs proves that they originate from the main cells of the kidney [58]. The engineered MSC-derived EVs that were designed with TRAIL (TNF-related apoptosis inducing ligand) shown a substantial effect on TRAIL-resistant renal carcinoma cell lines, such as RCC10 and HA7-RCC examples [59]. MSC-derived EVs have a moderate effect on renal cancer by promoting apoptosis and inhibiting proliferation. RCC is an important concern owing to its high metastatic rate, death rate, increased incidence, and resistance to treatment. It becomes difficult to diagnose solid tumours when there are unusual tumour cell patterns or insufficient tissue samples [60, 61].

EVs originating from ccRCC, papillary RCC (pRCC), and benign renal cell lines exhibit distinct signatures, enabling differentiation not only between RCC subtypes but also between RCC and benign renal cells [62]. EVs generated by ccRCC and pRCC, respectively, are specifically enriched in exosomal proteins, while EVs of benign renal cells, but not ccRCC, include

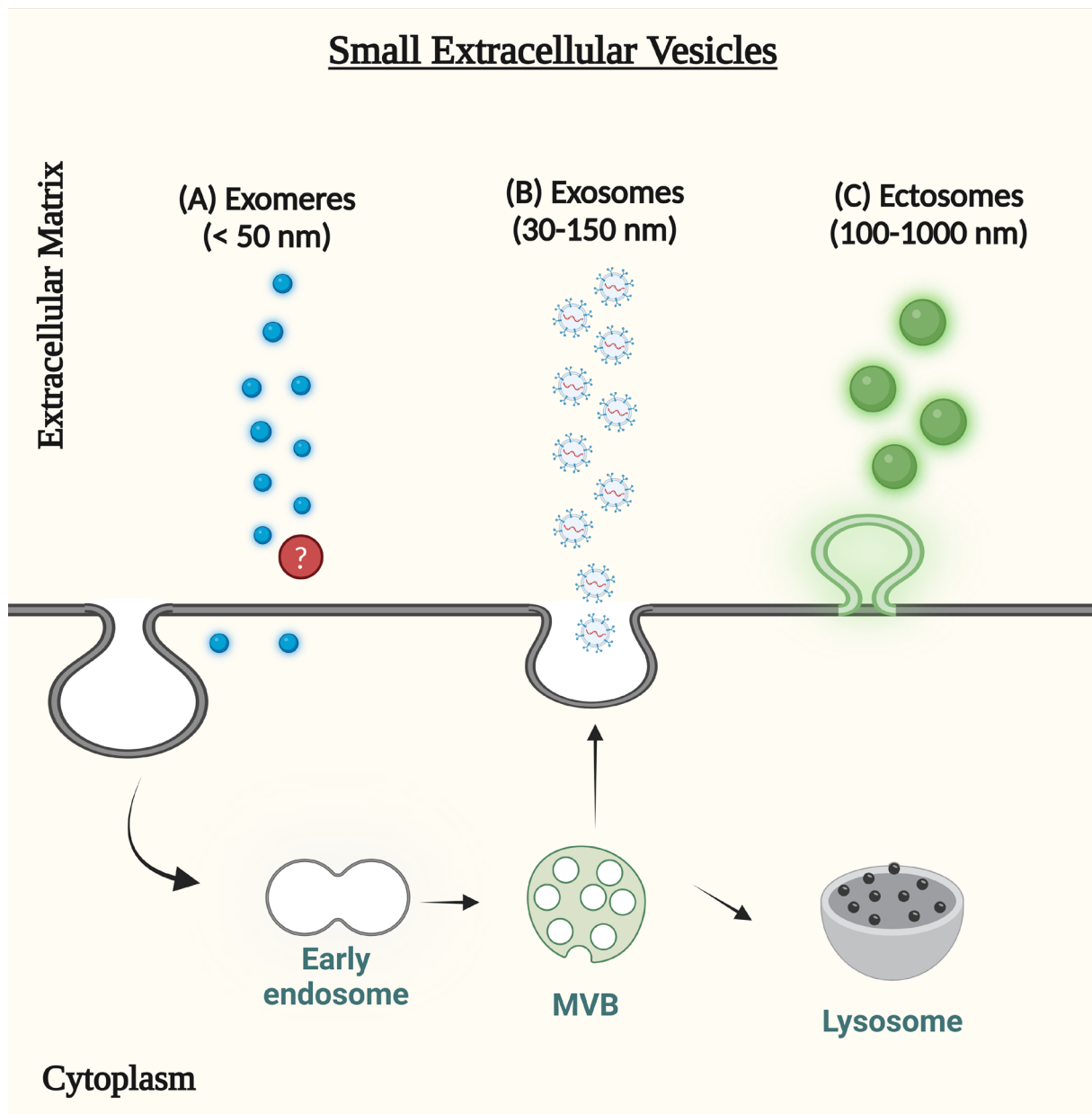


Figure 2 (A) Exomeres are extracellular vesicle-like non-membranous nanoparticles, but both their origin and the precise mechanisms underlying their production are still unclear (B) Endocytic vesicles merge with early endosomes to generate exosomes. Membrane budding creates intraluminal vesicles in the beginning endosomes. By fusing with the cell membrane, lumen vesicles can be destroyed by lysosomes or released into the extracellular environment. Released vesicles are called exosomes. (C) The outward protrusion of the plasma membrane releases ectosomes, often referred to as shedding microvesicles, into the extracellular matrix.

distinct exosomal mRNA of EPCAM, PRKCZ, PXDN, CXADR, EPS8L1, HOXA7, LAD1, MYO1D, ROCK2, and SLC35A3 [63]. Conversely, the epithelial tumour cell marker EpCAM is broadly expressed in both normal tubular and ccRCC samples. Furthermore, CDH2, COL7A1, FGFR2, BMPR1B, HDHD3, ICAM1, KIAA1462, and PFKFB4 mRNA are only present in EVs that are produced from ccRCCs [58, 63].

In addition to proteins and mRNAs, EVs are significantly enriched with non-coding RNAs, such as microRNAs (miRNAs), circular RNAs, and long non-coding RNAs (lncRNAs). Various miRNAs transported by EVs exhibit unique expression profiles

when comparing patients with RCC to individuals with good health. Grange and his fellows identified that within the CD105+ microvesicles, there was a notable upregulation of 24 miRNAs, such as miR-200c and miR-650, alongside a downregulation of 33 miRNAs, including miR-100 and miR-29 [64]. Furthermore, Zhang and his team observed that serum samples from patients with ccRCC displayed significantly heightened levels of exosomal miR-210 and miR-1233 in comparison to healthy controls, with these levels markedly diminishing following nephrectomy [65]. Therefore, the detection of exosomal miR-210 and miR-1233 in serum may prove to be a significant marker in the diagnosis

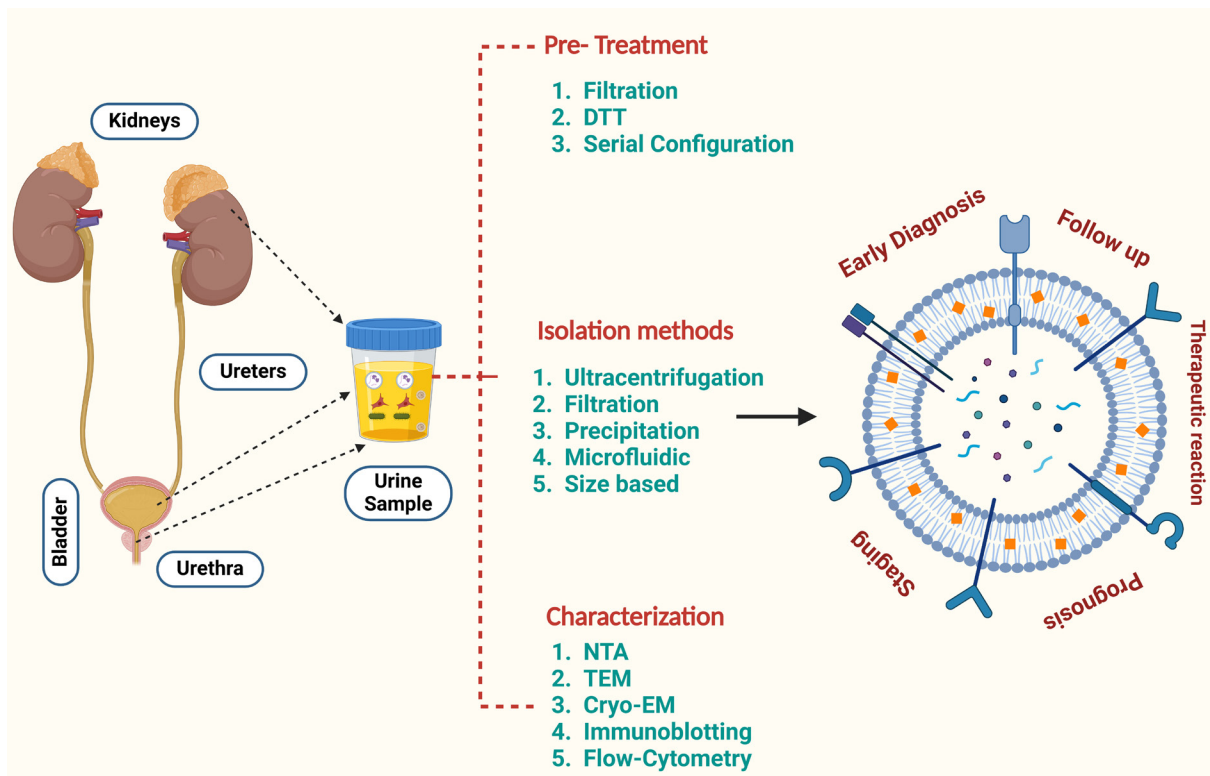


Figure 3. An overview of the processing stages for renal cancer biomarkers derived from uEVs, including miRNA and protein biomarkers.

and ongoing assessment of patients with ccRCC, especially in the context of liquid biopsies. Wang and his workfellow carried out research investigating serum exosomal miR-210, noting its upregulation in ccRCC, especially among patients presenting with advanced tumour stages, elevated Fuhrman grades, and the presence of metastases [66]. Fujii and colleagues performed an investigation into the serum levels of exosomal miR-224, emphasising its negative prognostic significance in patients with ccRCC. Their findings further elucidated the significant potential of exosomal miR 224 as a predictive biomarker for the detection of microinvasion or tumor metastasis subsequent to nephrectomy in patients with ccRCC [67].

Exosomal miR-21-5p derived from M2 macrophages has been linked to pro-metastatic effects in RCC via the activation of the PTEN/Akt pathway. A recent research revealed that the suppression of miR-21-5p within M2 exosomes led to a reduction in the metastatic capabilities of RCC cells [68]. Moreover, research has demonstrated that serum exosomes exhibit a markedly elevated gamma-glutamyl transferase (GGT) activity in individuals with advanced RCC, as well as those presenting with metastatic disease and microvascular invasion [69]. Hence, integrating exosomal GGT with standard diagnostic procedures may improve ccRCC diagnosis. Recent research has found higher exosomal MYO15A levels in the serum of patients with ccRCC, indicating a poorer prognosis and potential as a diagnostic target [70].

lncRNAs are RNA molecules that exceed 200 nucleotides in length and play a crucial role in regulating cellular processes, including transcription and protein translation, through their interactions with proteins, mRNAs, or miRNAs. They show certain expression patterns in tumor cells, which could make them useful for cancer diagnosis [71, 72]. Studies have shown that the transfer of lncARSR via exosomes enhances the expression of AXL and c-MET in RCC cells by competitively interacting with

miR-34/miR-449, thus playing a role in the emergence of resistance to sunitinib [73, 74]. Therefore, EVs are the most efficient means for transferring drug resistance in advanced RCC. These lncRNAs have the potential to be used as treatment strategies for chemotherapy resistance and prognostic indicators. Although there is potential, additional research is required to broaden and apply the use of EVs to identify new RCC biomarkers.

uEVs in renal carcinoma

Urine is the most readily available and simple body fluid for conducting studies on biomarkers related to kidney dysfunction. uEVs represent a diverse group primarily derived from the cells of the urogenital tract. The predominant sources of these vesicles include glomerular, tubular, prostate, and bladder cells [75]. uEVs have been the subject of extensive research because of their ability to represent kidney pathologies [76]. A proteomic survey revealed that 99.96% of the proteins found in uEVs are typical cells from the urogenital system. The fact that uEVs lack primarily basolateral markers but do express common apical membrane factors like transporters and channels indicates that they primarily derive from the apical surfaces of urogenital tissues [77]. An analysis of kidney-derived uEVs (collected from patients with nephrostomy catheters) with EVs from the entire urinary channel revealed that a significant amount of uEVs proteins existed throughout both kinds of specimens, indicating that the kidney is the principal producer of uEVs [78].

Tumor-derived EVs play a significant role in influencing the tumor's microenvironment, thereby sustaining, and promoting the growth of cancer [64]. Several specific markers of RCC were found in one of the first studies to compare the proteomic profiles of uEVs from patients with RCC and healthy individuals. These included decreased expression of neprilysin, extracellular matrix

metalloproteinase inducer (EMMPRIN; also known as basigin), dipeptidase 1, syntenin 1, and AQP1, and increased expression of metalloproteinase 9 (MMP9), ceruloplasmin, podocalyxin, carbonic anhydrase 9 (CAIX), and Dickkopf-related protein 4 (DKK4) [79]. A clinical study is currently underway to identify uEVs expressing CAIX in conjunction with exosomal marker CD9 in the urine of patients with ccRCC using electron microscopy [80].

Research has also explored uEVs miRNAs as potential biomarkers of RCC. It has been observed that individuals with ccRCC can be distinguished from healthy individuals by the presence of elevated levels of miR-126-3p in conjunction with miR-449a or miR-34b-5p in the cargo of uEVs [81]. Another research study indicated the elevation of miR-204-5p in urinary exosomes from patients with Xp11 translocation RCC, an extremely uncommon sporadic pediatric renal cancer, implying that this increase may serve as a valuable diagnostic for early diagnosis [82]. Metastatic RCC and poor survival rates are strongly linked to decreased miR-126 expression, which is induced by lncRNA DUXAP8 [83].

Kuczler et al. conducted a comparison analysis of exosomal mRNA in urine and tissue models from patients with RCC. Exosomal mRNA transcripts of ALOX5, RBL2, VEGFA, and TLK2 are particularly detected in the tissue and uEVs of patients with ccRCC [84]. In addition, patients with early stage ccRCC showed substantial downregulation of uEV-derived mRNA transcripts of NME2, AAMP, CAPNS1, VAMP8, and MYL12B [85]. miR-224-5p is highly elevated in both uEVs and tissues of patients with RCC compared with healthy controls. miR-224-5p stabilizes the expression of PD-L1 (programmed cell death protein 1) by directly inhibiting cyclin D1 (CCND1). This study clarified the mechanism by which miR-224-5p enhances resistance to T cell-dependent toxicity and metastasis by EV transmission among RCC cells [86].

Boccio et al. identified possible lipid biomarkers for RCC through the analysis of uEVs from RCC patients. These cancer-derived extracellular vesicles possess a distinctive lipidome comprising phosphatidylinositol phosphates (PIP), lysophospholipids (Lyso), phosphatidylethanolamines (PE), phosphatidylcholines (PC), mono-, di-, and triglycerides (MG/DG/TG), phosphatidic acids (PA), gangliosides (GL), and prostanoids (Pn) [87]. In conclusion, uEVs have demonstrated potential as the best option for minimally invasive, extremely sensitive, and unique renal cancer screening and treatment. **Figure 3** illustrates the methods for processing uEV-derived miRNA and protein indicators for renal malignancies.

Conclusion

Timely identification of RCC is crucial for enhancing patient survival, and uEVs present promising advantages in this context. The potential of uEV content in detecting cancer, prognosis, and monitoring, in addition to its probable therapeutic implications, makes it an attractive target for research in the development of new biomarkers for RCC. Furthermore, uEVs present a non-invasive and safer option than existing diagnostic and monitoring methods for RCC.

At present, research on uEVs is predominantly limited to preclinical studies and initial clinical trials, which face obstacles in the translation of experimental results into therapeutic applications. Additional thorough studies and clinical trials are necessary to enable the use of uEVs in clinical settings. Future studies should include a larger number of samples and a variety of tissue types, utilizing prospective study designs that provide stronger evidence and more reliable medical information to facilitate clinical translation. Moreover, the analysis of uEVs in relation to RCC has

been somewhat limited, and none of the identified molecules have been consistently validated in various studies. This highlights the need for further prospective clinical trials to identify reliable biomarkers.

In conclusion, uEVs are an appropriate medium for diagnosis because of their ease of collection and ability to represent the pathological conditions of the kidneys. Rapid urinary screening of predicted EV biomarkers could assist clinicians in diagnosis and facilitate the prompt selection of suitable therapies. Ultimately, its applications may have significant academic and commercial implications.

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Ethical policy

All procedures performed in this study were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Informed consent was obtained from all individual participants included in the study. Approval from institutional ethical committee was taken.

Availability of data and materials

All data generated or analysed during this study are included in this publication.

Author contributions

KG searched academic literature, wrote the draft manuscript, made the figures and submitted the final manuscript.

Competing interests

Authors report no conflict of interest.

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