Chapter 6

Mining Extracellular Vesicles for Clinically Relevant Noninvasive Diagnostic Biomarkers in Cancer

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Abstract

Extracellular vesicles (EVs) are nanosized vesicles secreted by virtually all cell types into the extracellular milieu. EVs transport bioactive molecules between cells and play multifaceted roles in cell-to-cell communications and in the pathogenesis of various human diseases including cancer. EVs are currently a focus of intensive interest, mainly because they hold a wealth of biological information in the form of differentially expressed nucleic acids and proteins, including DNA and cancer-related mutated genes, microRNAs, and a variety of transcriptional factors. Both the mutational content and any differentially expressed RNA are highly stable in patient blood or urine because they are encapsulated in EVs. This protects them against nuclease activity, pH change, temperature fluctuations, and multiple free-thaw cycles. Therefore, EVs isolated from patient fluids may serve as an ideal source of liquid biopsy for mining cancer signatures through mutation screening and genetic profiling. However, the methods for obtaining pure and intact EVs from patient samples, as well as the optimized characterization of tumor-derived EVs are still not rigorously defined for routine clinical use. High-throughput genomic or proteomic platforms may aid in the identification of novel diagnostic and prognostic biomarkers that collectively could lead to cancer monitoring and improved patient outcome.

Keywords: extracellular vesicles, exosomes, genetic profiling, biomarkers, noninvasive diagnosis, liquid biopsy, cancer

1. Introduction

Extracellular vesicles (EVs) are membrane-bounded nanosized vesicles secreted by almost every cell type studied to date. EVs carry a plethora of bioactive molecules comprising nucleic acids such as noncoding RNAs (ncRNAs), mRNA, and even DNA including both genomic

and mitochondrial DNA, lipids, carbohydrates, proteins, and variety of transcriptional factors [1–3]. Based on their size, morphology, and mode of release, EVs are broadly categorized into exosomes, microvesicles, and apoptotic bodies [4]. The best characterized EVs are exosomes and microvesicles. Exosomes are produced through the endocytic pathway followed by the fusion of the multivesicular bodies (MVBs) with the plasma membrane and are released into the extracellular environment. This involves several different components of sorting machinery taking place at endosomal compartments and the MVBs (reviewed elsewhere [4]). Conversely, microvesicles are shed directly from selective microdomains of the plasma membrane and involve several components of cytoskeleton machinery.

EVs were previously considered to be platelet-derived particles and were described as cellular dust or debris until they were first recognized as specific structures termed exosomes by Johnstone et al. in 1987 [5]. Initial studies by Raposo et al. [6], Zitvogel et al. [7], and Thery et al. [8] raised the new debate that EVs are not cellular dust and highlighted the functional importance of exosomes in immunological responses. In these studies, exosomes were reported to contain major histocompatibility complex class I and class II (MHC I, MHC II) that were efficiently able to induce T-cell responses. Ratajczak and colleagues further highlighted the biological significance of secreted vesicles [9], and they were the first to report that microvesicles contain mRNA that could be transferred horizontally to target cells and subsequently translated into corresponding proteins [10, 11]. A subsequent study by Deregibus et al. reported the horizontal transfer of mRNA that was biologically associated with the activation of angiogenic program in endothelial cells [12].

The first report that exosomes contain substantial amounts of microRNA (miRNA), mRNA, and small amounts of ribosomal RNAs was documented in 2007. Valadi et al. showed that the RNAs shuttled between cells as a novel mechanism of genetic exchange between cells [13]. Following this discovery, several other studies confirmed the presence of miRNA in vesicles and showed that their transfer to neighboring cells was functional [12, 14–16]. Valadi et al. also showed that heterologous transfer of mouse EV-mRNA to recipient human mast cells is translated into corresponding mouse proteins *in vitro*, indicating that EV-mediated transfer of mRNA can be functional in recipient cells [13]. Skog et al. provided a confirmatory study showing that EV-mediated transfer of mRNA could be translated into recipient cells, which further highlights the functional role of RNA transfer [15]. Later on, Pegtel et al. showed that miRNAs secreted by EBV-infected cells were transferred to uninfected recipient cells via EVs and could potentially repress the EBV target genes [16]. Additionally, EVs from dendritic cells (DCs) could fuse with autologous target DCs and efficiently release miRNA into recipient cell cytoplasm where they repress target mRNAs of acceptor DCs [17].

1.1. Biological functions of extracellular vesicles

At the present time, the biological functions of EVs are not fully understood in comparison to well-established, paracrine-secreted factors such as cytokines and hormones. Now EVs are gaining increased attention due to their novel role in the transport of various bioactive molecules that facilitate signal transduction between cells. The secreted EVs can be taken up directly by neighboring recipient cells or they may travel through biological fluids and

transport their cargo to distant organs in a paracrine manner. Considerable documented evidence emphasizes that EVs serve as mediators of cell-to-cell communication allowing the exchange of biological information between cells [18, 19]. EVs carrying diverse cargoes can move through biological fluids and thus may elicit long distance interorgan communication by dissemination of their cargo from one place to the other [20, 21]. It is possible that EVs may in fact mediate bidirectional communication and transport of regulatory molecules [22].

Due to their natural capacity in transportation and dissemination of abnormal proteins, lipids, mutated genes, and deregulated nucleic acids—EVs have been implicated in number of diseases such as neurodegenerative disease [23, 24], inflammatory and cardiovascular diseases [25, 26], and the development of cancers [15, 27–29]. The secretion and transportation of EVs from biologically active cells are likely to be context dependent, so that the signals that a particular cell receives may elicit tissue remodeling and regeneration as a response to diseases [30]. Moreover, the microbial EVs may mediate host-parasite interactions and progression of infectious diseases by disseminating virulence factors [31].

EVs derived from antigen presenting cells or cancer cells may also have a profound effect on immunomodulation, including both immune suppression and immune activation [30, 32, 33]. There is emerging role of EVs implicated in cellular differentiation, stem cell maintenance, and defining cell fates by facilitating the transmission of biological information from donor cells to recipient cells (reviewed elsewhere [22]). Such evolving roles of EVs is in part due to their abilities to mimic stem cell properties in promoting tissue's intrinsic regenerative programs and repair process within recipient cells in a paracrine manner [22]. Interestingly, the cargo of EVs is characteristic for their cell of origin, which presumably represents the disease-associated signature of their parent cells. The study of the cargo of EVs from different diseases could therefore be a rich resource for future biomarkers studies.

1.2. Extracellular vesicles as mediators of cancer initiation and metastasis

There is increased interest in how EVs may facilitate tumor progression. EVs secreted from cancer cells may carry oncogenic ncRNAs or mutated genes, which may induce aberrant gene regulation in recipient cells that induce tumor initiation [3, 14]. It is thought that EVs may educate certain recipient cells to take on a tumor-initiating phenotype. Such cells may then migrate to anatomically distinct locations leading to premetastatic colonization [28]. There are several mechanisms of tumor progression conferred by EVs including stromal remodeling, immune evasion, neovascularization, and metastasis [21, 30, 34–39]. The metastatic potential of EVs is in large part due to their ability to transmit abnormally expressed bioactive molecules such as oncoproteins, genomic and mitochondrial tumor DNA, transposon elements, and mutated genes to suitable recipient cells [14, 15, 27, 40–44].

There seems to be a strong association between EV-mediated transport of regulatory ncRNAs and the mediation of tumor initiation. EV-mediated delivery of miRNAs is thought to potentiate more diverse regulatory functions in comparison to EVs carrying other cargoes. This is mainly because EVs bearing miRNAs are capable of modulating genetic profiles of recipient cells and they may also be able to foster genomic instability [3]. It was recently shown

that astrocyte-derived EVs could mediate an intercellular transfer of PTEN-targeting miRNAs to recipient primary metastatic tumor cells in order to suppress PTEN expression, and thus allowing primary tumor cells to develop metastatic potential [45]. This supports the idea that EVs are able to shuttle miRNAs between tumor cells and their metastatic progenitors. Such a reciprocal cross-talk would confer a selective advantage by facilitating coevolution of primary tumors and also favor microenvironments for adaptive metastatic outgrowth. This process could also be helped by metabolic reprogramming of tumor microenvironment. For example, recently the miRNA signatures secreted from breast cancer cells were shown to facilitate metastasis by increasing nutrient availability and reprogramming the energy metabolism of nontumor cells in a given premetastatic niche [46]. Currently, long non-coding RNAs (lncRNAs) are increasingly being reported to contribute tumor initiation and metastasis [3] and are considered as extended messages in regulating responses to chemotherapy.

2. Methods

2.1. Diversity of extracellular vesicle sources

Biological fluids and cultured cell supernatant from *in vitro* systems offer a potential source for isolation of EVs; however, EVs isolated directly from body fluids are likely to be clinically more relevant. Blood plasma is the most commonly used source for EVs collection. According to a recent survey conducted by the International Society of Extracellular Vesicles (ISEV), the plasma (47%), serum (22%), urine (14%), cerebral spinal fluid (8%), milk (5%), and miscellaneous (4%) are the relative frequencies of body fluids analyzed [47]. The choice of selecting a certain physiological fluid depends on intended downstream analysis [4].

The diverse nature of biological fluids and the individual contaminants of each fluid may represent different molecular combinations outside EVs, and may thus require different isolation methods [48]. An important bottleneck is the lack of standardized methods for collection and processing of biofluids for isolation, purification, and separation of subpopulation of EVs with removed contaminants and retained integrity of EV-cargo prerequisite for intended downstream applications. The diverse nature of biological fluids suggests that EV cargo of each fluid may represent different composition and, therefore, a spectrum of methods will need to be considered to define contaminants of each fluid in order to obtain pure EVs.

2.1.1. Available techniques for EV isolation

There are a variety of methods available and more are being developed, some of them are poorly standardized. These include ultrafiltration, density gradient centrifugation, size exclusion chromatography and affinity isolation, polymeric precipitation, and the microfluidic devices [48]. Each method has variable isolation efficiencies when applied to different samples, such as blood plasma, milk, urine, and cell culture media.

A comparison of several conventional as well as high-throughput technologies for the isolation and characterization of different samples has been recently undertaken with a focus on their advantages and disadvantages [4]. Recently, the ISEV has made a critical analysis of various

techniques implemented for isolation of EVs and they have made potential recommendations [48, 49]. Differential ultracentrifugation remains the most widely used primary isolation method, comparable to several other techniques, and is suitable for large-volume isolations. However, for the isolation of EVs from low volume samples, it was found that size exclusion chromatography is now a more widely used technique, which allows separation of EV from the bulk of soluble proteins. In this method, the separation is purely based on particle size, therefore contaminating particles in the EV size range such as lipoprotein complexes may be coisolated [48, 50]. When the intent is to capture a selective class of EVs, immunoaffinity capture offers an alternative method with a much higher selective specificity. The method can yield pure EV subpopulations, but is highly influenced by both the choice of affinity reagent and the ligand density on different EV types [48].

Other methods include microfluidic devices, filtration, and various commercially available kits. The commercial kits often make the use of volume-excluding polymers, such as polyethylene glycol (PEG), which enables rapid EV isolation from culture media or from body fluids. However, such polymers may also coprecipitate protein complexes that can contaminate EV isolates. Therefore, the ISEV has recommended using two different EV isolation techniques that are based on distinct principles of separation and each will thus enrich for different subpopulations of vesicles. Since each method potentially coisolates the contaminants, such as protein complexes and lipoproteins to different degrees, the ISEV proposes that a combination of techniques be applied, such as density gradient centrifugation followed by size exclusion or immunoaffinity capture [48].

The method of choice should take into account several factors: sample type, volume, yield, integrity, purity of EVs required for specific downstream analysis, as well as the available instrumentation and processing time [4]. The chosen method will be influenced by whether the sample is derived from cell-culture media or from body-fluids and whether the intended analysis is proteomic or genomic. Therefore, the choice of different isolation methods for EVs will impact the amount, type, and purity of EVs recovered and will guide the type of downstream analysis of EVs that is the most practical.

2.2. Characterization of EVs and available analytical technologies

After the isolation procedures, one needs to characterize EVs for their size determination, detection of common EVs marker, morphology, and concentration (quantification), for which there is a variety of techniques available [4, 51, 52]. Characterization of EVs currently presents various challenges, mainly due to their small size, the complexity of the EV cargo, and the physical parameters of available instruments for measuring nanosized EVs. Given the fact that EVs are isolated from a variety of different sources with highly variable composition (as stated above), it is difficult to provide general recommendations for EV isolation and characterization. The sample type, sample volume, and the choice of downstream application all will be an influence on the characterization instruments employed.

The mean size and overall size distribution of individual subpopulations of EVs, as well as their relative abundance, can be determined by nanotracking analysis (NTA), Zetaview [53–60], and tunable resistive pulse sensing (qNANO) [61]. Electron microscopy is used to assess

the submicron phenotype of EVs [51, 59, 62–64], whereas flow cytometry is used for enumerating, phenotyping, and sorting of EVs based on their size distribution [65, 66]. Western blotting is implemented when the aim is to detect EV-markers such as CD63, CD81, and CD9. A recent survey conducted by ISEV has mentioned that the three most widely used techniques for EVs characterization are Western blotting (74%), single-particle tracking (SPT, 72%), and electron microscopy (60%) [47].

Hitherto, the flow cytometry remains a popular tool for measuring EVs [65, 66]; however, fundamental principles and limitations of the instrument need to be considered [67]. EVs isolated by ultracentrifugation may cause aggregation of EVs thus rendering subsequent difficulties for flow cytometric analysis or single particle tracking analysis [47], whereas, those isolated using size exclusion kits may lead to subsequent difficulties for downstream EV analysis by Western blotting. Recently, tunable resistive pulse sensing has been used to precisely determine the concentration of EVs. Further validations are required to show that this promising new method is reproducible and widely applicable for characterizing EVs [61].

The characterization of heterogeneous subpopulations of EVs into their component parts remains an unresolved issue. This problem of EV subclassification arises because almost all subtypes, including exosomes and microvesicles, share same/common EV detection markers such as CD63, CD81, and CD9 [48, 52]. Recent papers claim successful subclassification EVs based on general surface protein profiling (proteomics) [57, 68] or on RNA content profiling (RNAomics) of individual EV populations [69–73]. New advances in both EV isolation techniques and detailed optimization and standardization of existing techniques or protocols will facilitate progress toward more precise and reliable EV characterization. These crucial steps will greatly influence the identification of specific biomarkers in EV subpopulations.

2.2.1. Characterization and profiling of nucleic acid content from EVs: potential issues

Prior to profiling of RNA content from EVs, it is important to assess the quality of RNA. There are predominantly two populations of secreted extracellular RNA (exRNA) either in association with RNA-binding proteins, or as a part of lipoprotein complexes, or alternatively vesicle bound RNAs. It is thought that such protein complexes might be coprecipitated and coisolated along with EVs during ultra-centrifugation. Therefore, the methods that remove contaminating proteins from EV aggregates are highly required.

The occurrence of extracellular RNA (exRNA) either inside secreted EVs or outside EVs (i.e., non-EV exRNA) is a controversial subject at the present time, as there are discrepancies in the results shown by different laboratories [74–78]. In order to discriminate RNA encapsulated within/or on the surface of EVs from those non-EV bound exRNA, it is critical to digest isolated RNA fractions with RNase and proteinase to disrupt the ribonucleoproteins and any RNA exterior to vesicles [48]. This procedure will deplete non-EV exRNA leaving behind EV-encapsulated RNA.

A potential issue in studies using *in vitro* methods is the fetal bovine serum (FBS) that is used for cell cultures. FBS already contains various bovine RNA species that are retained even after extended period of ultracentrifugation during the preparations of the vesicle-depleted FBS [79]. This raises the possibility that the RNA being analyzed might not be exclusively from

human vesicles and subsequent qPCR or sequencing analysis may contain artifactual bovine nucleic acids derived from nonvesicular bound RNA, which may bias results [80].

An additional issue related to expression analysis, and sequencing library preparations from EV-RNA; is the low input sample material. Particularly this refers to small sample source e.g., less EV-RNA from low volume of patient blood or the usage of low recovery protocol). Low input material may suffer biases not only when the library is prepared but also during the EV-RNA ligation step. Measuring the quantity and integrity of EV-associated RNA is challenging due to limited amount of RNA available and the lack of reference standards, such as those established for cellular mRNA. Recently, the ISEV has addressed these issues and have made recommendations for the assessment and analysis of the nature of EV-associated RNAs [48]. Detection of the levels of certain transcripts by highly sensitive RT-qPCR may be used as a proxy for total RNA quantification in samples containing a low abundance of RNA.

Sensitive techniques, such as Agilent Bioanalyzer pico chip and the Quant-iT RiboGreen RNA Assay, have been proven to be more suitable methods for the quantification EV-RNA than that of Nanodrop method. Most of the techniques (with the exception of the Qubit RNA HS Assay) are thought to be sensitive to DNA contamination. Therefore, ISEV recommends pretreatment of samples with DNase for accurate RNA quantitation [48]. Due to the enrichment of small RNA species in EVs, the main focus of recent studies is the assessment of miRNAs and other small ncRNAs. Such studies take into consideration subsequent expression analyses and deep sequencing experiments. ISEV has provided recommendations on the different steps of EV-RNA analysis, such as RT-qPCR analysis, selection of reference genes, deep sequencing, library preparation, biases issues, data normalization, and bioinformatics analysis.

3. Genetic profiling of EVs from cancer-derived biofluids: a stable source of noninvasive diagnosis

In the absence of early symptoms, most cancers are diagnosed at an advanced stage, by which time patients have poor outcomes and tumors have often metastasized. Pathological evaluations and resulting treatment approaches are often determined based on biopsy material. The detection of biomarkers from body fluids may offer significant advantage over the use of tissue markers, because biopsies are invasive procedures and are associated with bleeding and risk of infections. Moreover, biopsies are often difficult to perform for organs that lie deep within the body and may suffer from sample bias [81].

There is an intensive interest in mining biological fluids as a noninvasive source of biomarkers detection. For example, elevated prostate-specific antigen (PSA) levels in plasma are routinely used for the early detection and monitoring of prostate cancer. However, since the blood levels of this assay vary widely, PSA testing often fails to detect a new cancer or to accurately predict disease stage. Novel more precise blood and urine biomarkers are needed at both diagnosis and during the disease progression of prostate cancer [82]. The expression levels of miRNAs in prostate cancer show considerable promise as potential biomarkers with clinical applications. Since the miRNA content of EVs reflects the miRNA expression profile of the cells they originated from, there has been considerable interest in mining miRNAs from EVs

in prostate cancer. One good example is miR-16, which has increased expression in plasma from metastatic prostate cancer patients, but has reduced levels of expression in both primary and metastatic prostate cancer tissue RNA samples [83]. In contrast, miR-21 was found to be elevated in the early disease, but not in advanced prostate cancer [84]. One of the most powerful applications could be early detecting of prostate cancer in patients' urine. An agglutination methods were used recently to isolate miRNAs from prostate cancer urine, which showed that upregulation of miR-574-3p, miR-141-5p, and miR-21-5p was associated with disease [85].

Since deregulated miRNA expression is an early event in tumorigenesis, measuring circulating miRNA levels could be potentially useful for early cancer detection, and may contribute to greatly measure the success of treatment or evaluate the therapeutic response.

4. Mining extracellular vesicles for cancer diagnosis

Proteins and nucleic acids encapsulated within EVs circulating in body fluids are thought to be more stable against proteases and nucleases that are naturally present in body fluids. The protection of nucleic acids in EVs provides a great advantage of storage conditions as well as handling at adverse physical conditions such as fluctuations in temperature and changes in pH, multiple freeze, and thaw cycles, and thus could be an appealing source for biomarker development [4]. Moreover, circulating EVs from cancer patients have been found to express signatures that are significantly distinct from profiles of benign disease or normal controls [86–88].

Interestingly, tumor cells release EVs containing tumor-specific content that could be easily isolated from various body fluids such as blood plasma, serum, and urine. In this regard, EV-assisted liquid biopsies offer an inherent advantage, mainly because samples could be collected longitudinally with great ease and in large quantities (i.e., sample reproducibility). EVs release is an active process and tumor cells can shed plenty of EVs per milliliter of plasma [89]. EVs released into body fluids containing differentially or aberrantly expressed miRNAs often retain the characteristics of the tumors from which they originated [15, 87, 90]. Interestingly, it has been proposed that the cellular origin of EVs from certain cancer types or cell types could be used to determine the likely identity of an unknown disease [91]. This may assist the assessment of EVs in a body fluid from new cancer patients when there is uncertainty about the type of tumor. Consequently, EVs from biological fluids could be subjected to large-scale screening of tumor-specific markers [33, 40, 89, 92–95].

The comprehensive detection of diverse EV components, in particular the profiling of EV-linked ncRNAs including short ncRNAs such as miRNAs, as well as lncRNAs from peripheral blood or urine of cancer patients, may be a source of predictive cancer signatures for early diagnosis of specific cancer types. There is already evidence for using exRNA including vesicle bound and nonvesicle bound exRNA comprising circulatory miRNAs and lncRNAs from human body fluids for determining diagnostic and prognostic value of RNA signatures related to cancer [3]. In this context, the presence of ncRNA in EVs may serve an additional platform for biomarker discovery. The global profiling of EV-encapsulated RNAs with mutation characterization and/or without mutation characterization [33] could serve as

a potential source for cancer detection. This could allow clinicians to perform patient stratification (companion diagnostics), screening, monitoring treatment response, and detection of minimal residual disease after surgery/recurrence [89].

miRNA profiling from EpCAM-positive EVs from serum of ovarian cancer patients demonstrated that EV-derived miRNA signatures from advanced stage patients are significantly distinct from those at benign stage, providing a possible biomarker signature to distinguish early stage cancer from advanced disease [87]. Expression of candidate cell-free urine miRNAs in ovarian cancer and endometrial cancer patients has demonstrated their prospective use as biomarkers [96]. However, in order to confirm the diagnostic potential of urine miRNAs in gynecological cancers, it requires large number of clinical samples and large-scale expression profiling studies. Additionally, mining EVs from urine samples of cancer patients may help early diagnosis. In this context, the most often studied cancer for mining urinary EVs is the prostate cancer [97–107].

In addition to their utility in diagnostic platforms, the EV-associated cargo from serum/plasma may also serve as bona fide signatures of disease prognosis, tumor recurrence, and overall survival. This refers to prognostic implications against chemotherapies as well as radiotherapies related to several cancer types. Therefore, the ncRNA signatures from EV sources may have clinical implications. However, it is critical to compare and standardize results of global investigations regarding EV-associated circulating ncRNAs as well as the recommendations for preanalytic considerations in biomarker discovery.

Although, the biological consequences of the discriminatory distribution of miRNAs in EVs are not fully known, it is possible that measuring the concentration of miRNAs from biological fluids (i.e., blood, urine serous, and ascites) and making corresponding comparisons could allow biomarker identification. Interestingly, the miR-21, one the global tumor marker, is found in EVs from serum and plasma of various cancer types and may serve as an independent marker of tumor diagnosis and prognosis [33, 108–110]. Presumably, the global profiling or selective screening of EV-RNAs against mutations may predict tumor-specific signature, whereas the enrichment on ncRNAs within tumor cell-derived EVs could offer a promising platform for developing disease biomarkers.

Another area of interest for EVs in cancer is their potential to restore gene activity that has been lost. PTEN is frequently deleted in prostate cancer and associated with aggressive disease [111]. Using an *in vitro* system it was shown that through EVs the PTEN can be transferred back into cells that have lost PTEN expression [82]. Interestingly, the transferred PTEN was competent to confer tumor suppression, suggesting that exosomal PTEN may in the future be able compensate for PTEN loss in PTEN-deficient prostate cancers. Initially, it was shown that cancer cells release PTEN via EVs and could be transferred to other cells through EVs [112]. In cells that exhibit a reduction of PTEN expression or complete loss of PTEN expression, the tumor-suppression activity was restored via EV-mediated transfer of PTEN to acceptor cells. Interestingly, PTEN could be detected from EVs that circulate in the blood of prostate cancer patients. Conversely, the normal subjects have no PTEN expression in their blood EVs. Moreover, the prostate-specific antigen (PSA) was also detected in EVs derived from prostate cancer patients. These data suggest that EV-associated PTEN can not only compensate for PTEN loss in PTEN deficient cells, but also may have diagnostic value for prostate cancer [112]. The workflow to mining EVs for nucleic acid analysis and proteomic profiling is given in **Figure 1**.

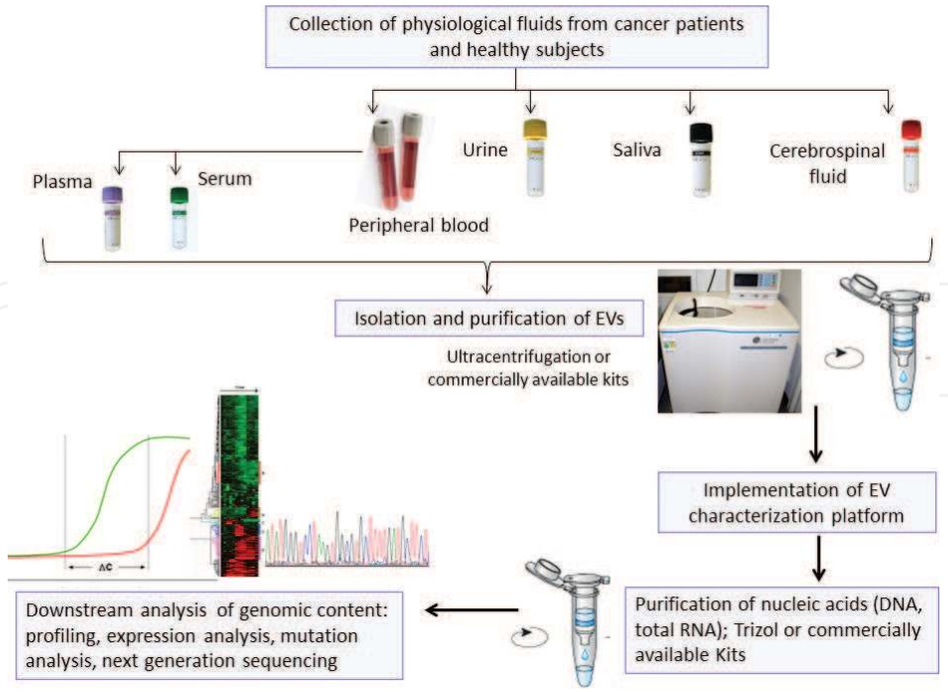

Figure 1. A flow sheet of extracellular vesicle (EV) isolation and analysis: for utilizing EVs as biomarkers, the physiological fluids from cancer patients and healthy subjects are collected, and EVs are isolated from various fluids. EVs from each source need to be characterized and EV-RNA is isolated for downstream analysis in order to identify genetic aberrations or profiling of genomic content.

5. Conclusion

There is still a long way to go to fully understand EV content in the context of cancer as a systemic disease. It will be necessary to establish a link between tumor occurrence, progression, treatment response, and corresponding changes in EV content. Since biopsies are associated with potential risk factors such as surgical resection and associated toxicity, alternative methods will be required for early diagnosis. The longitudinal collection of EVs from patient body fluids may offer untapped source for liquid biopsy. As such, the EVs cargo itself may represent an attractive source of multiple candidate biomarkers that could provide clinically useful information for cancer management. The ease with which EVs can be collected and purified from body fluids suggests that biomarkers present in their cargo could eventually be part of personalized cancer care, possibly replacing more invasive biopsies.

The detection of candidate molecules anchored to circulating EVs, may thus allow cancers to be identified from several drops of a patient blood, and may serve as highly sensitive screening tools [113]. Therefore, EVs are ideal source of screening intact molecular signatures of tumor origin. EV-associated ncRNAs including miRNAs as well as lncRNAs are currently the most frequently exploited biomarkers for cancer diagnosis [3]. Identification of aberrantly expressed RNA molecules, mutated genes, or proteins in EVs from body fluids of cancer patients can be subjected to next-generation genomics and proteomics approaches that may aid in the identification of diagnostic and prognostic biomarkers.

To be able to use EVs as liquid biopsies, a comprehensive inventory of their constituents such as proteins, DNA, RNA, and metabolites, followed by the validation of distinct candidates in the frame of a multicenter clinical study is required (see workflow for biomarker development [4]). However, the noninvasive detection technologies should be accurate, fast, and potentially inexpensive.

It has been argued that the development of high-throughput approaches and robust capture platforms will warrant the implications of EVs in routine biomarker development, and therapeutic implications with a proposed workflow sheet to be applied for US Food and Drug Administration (FDA) approval [4]. What needs to be considered as a priority is the standardization of scientific reporting. At present, differences in reporting approaches could make it difficult to compare and standardize the potential therapeutic effects of EVs. Such inconsistencies may limit the likelihood of translating EVs into human clinical trials. Efforts are being made to ensure transparent reporting of EV findings in order to facilitate interpretation and replication of experiments [114]. This will help to put experimental guidelines into practice. Since there is intensive interest in the field both in basic research as well as therapeutic point of view, it is anticipated that in the next decade, EVs arena will see significant advances in clinical pipelines [32].

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Conflict of interest

The authors of this chapter declare that there is no conflict of interest.

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References

[1] Keerthikumar S, et al. ExoCarta: A web-based compendium of exosomal cargo. Journal of Molecular Biology. 2016;**428**(4):688-692

[2] Kim DK. et al. EVpedia: A community web portal for extracellular vesicles research. Bioinformatics. 2015;**31**(6):933-939

[3] Fatima F, Nawaz M. Vesiculated long non-coding RNAs: Offshore packages deciphering Trans-Regulation between cells, cancer progression and resistance to therapies. Non-Coding RNA. 2017;**3**(1):10

[4] Nawaz M, et al. The emerging role of extracellular vesicles as biomarkers for urogenital cancers. Nature Reviews Urology 2014;**11**(12):688-701

[5] Johnstone RM, et al. Vesicle formation during reticulocyte maturation. Association of plasma membrane activities with released vesicles (exosomes). Journal of Biological Chemistry 1987;**262**(19):9412-9420

[6] Raposo G, et al. B lymphocytes secrete antigen-presenting vesicles. Journal of Experimental Medicine. 1996;**183**(3):1161-1172

[7] Zitvogel L, et al. Eradication of established murine tumors using a novel cell-free vaccine: Dendritic cell-derived exosomes. Nature Medicine. 1998;**4**(5):594-600

[8] Thery C, et al. Molecular characterization of dendritic cell-derived exosomes. Selective accumulation of the heat shock protein hsc73. Journal of Cell Biology. 1999;**147**(3):599-610

[9] Ratajczak J, et al. Biological significance of the different erythropoietic factors secreted by normal human early erythroid cells. Leukemia and Lymphoma. 2003;**44**(5):767-774

[10] Ratajczak J, et al. Embryonic stem cell-derived microvesicles reprogram hematopoietic progenitors: Evidence for horizontal transfer of mRNA and protein delivery. Leukemia. 2006;**20**(5):847-856

[11] Baj-Krzyworzeka M, et al. Tumour-derived microvesicles carry several surface determinants and mRNA of tumour cells and transfer some of these determinants to monocytes. Cancer Immunology, Immunotherapy. 2006;**55**(7):808-818

[12] Deregibus MC, et al. Endothelial progenitor cell derived microvesicles activate an angiogenic program in endothelial cells by a horizontal transfer of mRNA. Blood. 2007;**110**(7):2440-2448

[13] Valadi H, et al. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. Nature Cell Biology. 2007;**9**(6):654-659

[14] Al-Nedawi K, et al. Intercellular transfer of the oncogenic receptor EGFRvIII by microvesicles derived from tumour cells. Nature Cell Biology. 2008;**10**(5):619-624

[15] Skog J, et al. Glioblastoma microvesicles transport RNA and proteins that promote tumour growth and provide diagnostic biomarkers. Nature Cell Biology. 2008;**10**(12):1470-1476

[16] Pegtel DM, et al. Functional delivery of viral miRNAs via exosomes. Proceedings of the National Academy of Sciences. 2010;**107**(14):6328-6333

[17] Montecalvo A, et al. Mechanism of transfer of functional microRNAs between mouse dendritic cells via exosomes. Blood. 2012;**119**(3):756-766

[18] Mathivanan S, Ji H, Simpson RJ. Exosomes: Extracellular organelles important in intercellular communication. Journal of Proteomics. 2010;**73**(10):1907-1920

[19] Raposo G, Stoorvogel W. Extracellular vesicles: Exosomes, microvesicles, and friends. Journal of Cell Biology. 2013;**200**(4):373-383

[20] Thomou T, et al. Adipose-derived circulating miRNAs regulate gene expression in other tissues. Nature. 2017;**542**(7642):450-455

[21] Hoshino, A., et al. Tumour exosome integrins determine organotropic metastasis. Nature. 2015;**527**(7578):pp. 329-335.

[22] Nawaz M, et al. Extracellular vesicles: Evolving factors in stem cell biology. Stem Cells International. 2016;**2016**:1073140

[23] Rajendran L, et al. Alzheimer's disease beta-amyloid peptides are released in association with exosomes. Proceedings of the National Academy of Sciences. 2006;**103**(30): 11172-11177

[24] Vella LJ, et al. The role of exosomes in the processing of proteins associated with neurodegenerative diseases. European Biophysics Journal. 2008;**37**(3):323-332

[25] Buzas EI, et al. Emerging role of extracellular vesicles in inflammatory diseases. Nature Reviews Rheumatology. 2014;**10**(6):356-364

[26] Osteikoetxea X, et al. Extracellular vesicles in cardiovascular disease: Are they Jedi or Sith?. Journal of Physiology. 2016;**594**(11):2881-2894

[27] Al-Nedawi K, et al. Endothelial expression of autocrine VEGF upon the uptake of tumor-derived microvesicles containing oncogenic EGFR. Proceedings of the National Academy of Sciences. 2009;**106**(10):3794-3799

[28] Peinado H, et al. Melanoma exosomes educate bone marrow progenitor cells toward a pro-metastatic phenotype through MET. Nature Medicine. 2012;**18**(6):883-891

[29] Nawaz M, et al. Microvesicles in gliomas and medulloblastomas: An overview. Journal of Cancer Therapy. 2014;**5**(2):10

[30] Fatima F, Nawaz M. Stem cell-derived exosomes: Roles in stromal remodeling, tumor progression, and cancer immunotherapy. Chinese Journal of Cancer. 2015;**34**(12):541-553

[31] Schwab A, et al. Extracellular vesicles from infected cells: Potential for direct pathogenesis. Frontiers in Microbiology. 2015;**6**:1132

[32] Fatima F, Nawaz M. Nexus between extracellular vesicles, immunomodulation and tissue remodeling: For good or for bad? Annals of Translational Medicine. 2017;**5**(6):139

[33] Nawaz M, et al. Extracellular vesicles in ovarian cancer: Applications to tumor biology, immunotherapy and biomarker discovery. Expert Review of Proteomics. 2016; **13**(4):395-409

[34] Janowska-Wieczorek A, et al. Microvesicles derived from activated platelets induce metastasis and angiogenesis in lung cancer. International Journal of Cancer. 2005; **113**(5):752-760

[35] Janowska-Wieczorek A, et al. Enhancing effect of platelet-derived microvesicles on the invasive potential of breast cancer cells. Transfusion. 2006;**46**(7):1199-1209

[36] Costa-Silva B, et al. Pancreatic cancer exosomes initiate pre-metastatic niche formation in the liver. Nature Cell Biology. 2015;**17**(6):816-826

[37] Lima LG, et al. Tumor-derived microvesicles modulate the establishment of metastatic melanoma in a phosphatidylserine-dependent manner. Cancer Letters. 2009;**283**(2): 168-175

[38] Greening DW, et al. Emerging roles of exosomes during epithelial-mesenchymal transition and cancer progression. Seminars in Cell and Developmental Biology. 2015;**40**:60-71

[39] Taverna S, et al. Exosomal shuttling of miR-126 in endothelial cells modulates adhesive and migratory abilities of chronic myelogenous leukemia cells. Molecular Cancer. 2014;**13**:169

[40] Balaj L, et al. Tumour microvesicles contain retrotransposon elements and amplified oncogene sequences. Nature Communications. 2011;**2**:180

[41] Guescini M, et al. Astrocytes and Glioblastoma cells release exosomes carrying mtDNA. Journal of Neural Transmission (Vienna). 2010;**117**(1):1-4

[42] Greening DW, et al. Secreted primary human malignant mesothelioma exosome signature reflects oncogenic cargo. Science Report. 2016;**6**:32643

[43] Di Vizio D, et al. Large oncosomes in human prostate cancer tissues and in the circulation of mice with metastatic disease. American Journal of Pathology. 2012;**181**(5):1573-1584

[44] Morello M, et al. Large oncosomes mediate intercellular transfer of functional microRNA. Cell Cycle. 2013;**12**(22):3526-3536

[45] Zhang L, et al. Microenvironment-induced PTEN loss by exosomal microRNA primes brain metastasis outgrowth. Nature. 2015;**527**(7576):100-104

[46] Fong MY, et al. Breast-cancer-secreted miR-122 reprograms glucose metabolism in premetastatic niche to promote metastasis. Nature Cell Biology. 2015;**17**(2):183-194

[47] Gardiner C, et al. Techniques used for the isolation and characterization of extracellular vesicles: Results of a worldwide survey. Journal of Extracellular Vesicles. 2016;**5**:32945

[48] Mateescu B, et al. Obstacles and opportunities in the functional analysis of extracellular vesicle RNA – an ISEV position paper. Journal of Extracellular Vesicles. 2017;**6**(1):1286095

[49] Witwer KW, et al. Standardization of sample collection, isolation and analysis methods in extracellular vesicle research. Journal of Extracellular Vesicles. 2013;**2**

[50] Sodar BW, et al. Low-density lipoprotein mimics blood plasma-derived exosomes and microvesicles during isolation and detection. Science Report. 2016;**6**:24316

[51] Rupert DL, et al. Methods for the physical characterization and quantification of extracellular vesicles in biological samples. Biochimica et Biophysica Acta. 2017;**1861**(1 Pt A):3164-3179

[52] Maas SL, et al. Possibilities and limitations of current technologies for quantification of biological extracellular vesicles and synthetic mimics. Journal of Controlled Release. 2015;**200**:87-96

[53] Baldwin S, et al. Analyzing the miRNA content of extracellular vesicles by fluorescence nanoparticle tracking. Nanomedicine. 2016;**13**(2):765-770

[54] Dragovic RA, et al. Sizing and phenotyping of cellular vesicles using Nanoparticle Tracking Analysis. Nanomedicine. 2011;**7**(6):780-788

[55] Gardiner C, et al. Extracellular vesicle sizing and enumeration by nanoparticle tracking analysis. Journal of Extracellular Vesicles. 2013;**2**

[56] Pachler K, et al. A Good Manufacturing Practice-grade standard protocol for exclusively human mesenchymal stromal cell-derived extracellular vesicles. Cytotherapy. 2017;**19**(4):458-472

[57] Kibria G, et al. A rapid, automated surface protein profiling of single circulating exosomes in human blood. Science Report. 2016;**6**:36502

[58] Nardi Fda S, et al. High levels of circulating extracellular vesicles with altered expression and function during pregnancy. Immunobiology. 2016;**221**(7):753-760

[59] van der Pol E, et al. Particle size distribution of exosomes and microvesicles determined by transmission electron microscopy, flow cytometry, nanoparticle tracking analysis, and resistive pulse sensing. Journal of Thrombosis and Haemostasis. 2014;**12**(7):1182-1192

[60] Dragovic RA, et al. Isolation of syncytiotrophoblast microvesicles and exosomes and their characterisation by multicolour flow cytometry and fluorescence nanoparticle tracking analysis. Methods. 2015;**87**:64-74

[61] Vogel R, et al. A standardized method to determine the concentration of extracellular vesicles using tunable resistive pulse sensing. Journal of Extracellular Vesicles. 2016;**5**:31242

[62] Fertig ET, Gherghiceanu M, Popescu LM. Extracellular vesicles release by cardiac telocytes: Electron microscopy and electron tomography. Journal of Cellular and Molecular Medicine. 2014;**18**(10):1938-1943

[63] Brisson AR, et al. Extracellular vesicles from activated platelets: A semiquantitative cryo-electron microscopy and immuno-gold labeling study. Platelets. 2017;**28**(3):263-271

[64] Hoog JL, Lotvall J. Diversity of extracellular vesicles in human ejaculates revealed by cryo-electron microscopy. Journal of Extracellular Vesicles. 2015;**4**:28680

[65] Groot Kormelink T, et al. Prerequisites for the analysis and sorting of extracellular vesicle subpopulations by high-resolution flow cytometry. Cytometry A. 2016;**89**(2):135-147

[66] van der Vlist EJ, et al. Fluorescent labeling of nano-sized vesicles released by cells and subsequent quantitative and qualitative analysis by high-resolution flow cytometry. Nature Protocols. 2012;**7**(7):1311-1326

[67] Nolan JP. Flow cytometry of extracellular vesicles: Potential, pitfalls, and prospects. Current Protocols in Cytometry. 2015;**73**:13 14 1-16

[68] Kowal J, et al. Proteomic comparison defines novel markers to characterize heterogeneous populations of extracellular vesicle subtypes. Proceedings of the National Academy of Sciences. 2016;**113**(8):E968-E977

[69] Lasser C, et al. Two distinct extracellular RNA signatures released by a single cell type identified by microarray and next-generation sequencing. RNA Biology. 2017;**14**(1):58-72

[70] Lunavat TR, et al. Small RNA deep sequencing discriminates subsets of extracellular vesicles released by melanoma cells--Evidence of unique microRNA cargos. RNA Biology. 2015;**12**(8):810-823

[71] Crescitelli R, et al. Distinct RNA profiles in subpopulations of extracellular vesicles: Apoptotic bodies, microvesicles and exosomes. Journal of Extracellular Vesicles. 2013;**2**

[72] Conley A, et al. High-throughput sequencing of two populations of extracellular vesicles provides an mRNA signature that can be detected in the circulation of breast cancer patients. RNA Biology. 2016;**14**(3):305-316

[73] Huang X, et al. Characterization of human plasma-derived exosomal RNAs by deep sequencing. BMC Genomics. 2013;**14**:319

[74] Arroyo JD, et al. Argonaute2 complexes carry a population of circulating microRNAs independent of vesicles in human plasma. Proceedings of the National Academy of Sciences. 2011;**108**(12):5003-5008

[75] Turchinovich A, et al. Characterization of extracellular circulating microRNA. Nucleic Acids Research. 2011;**39**(16):7223-7233

[76] Gourzones C, et al. Consistent high concentration of the viral microRNA BART17 in plasma samples from nasopharyngeal carcinoma patients--evidence of non-exosomal transport. Virology Journal. 2013;**10**:119

[77] Chevillet JR, et al. Quantitative and stoichiometric analysis of the microRNA content of exosomes. Proceedings of the National Academy of Sciences 2014;**111**(41):14888-14893

[78] Patton JG, et al. Biogenesis, delivery, and function of extracellular RNA. Journal of Extracellular Vesicles. 2015;**4**:27494

[79] Wei Z, et al. Fetal bovine serum RNA interferes with the cell culture derived extracellular RNA. Science Report. 2016;**6**:31175

[80] Tosar JP, et al. Ribonucleic artefacts: Are some extracellular RNA discoveries driven by cell culture medium components? Journal of Extracellular Vesicles. 2017;**6**(1):1272832

[81] Bedard PL, et al. Tumour heterogeneity in the clinic. Nature. 2013;**501**(7467):355-364

[82] Valentino A, et al. Exosomal microRNAs in liquid biopsies: Future biomarkers for prostate cancer. Clinical and Translational Oncology. 2017;**19**(6):651-657

[83] Bonci D, et al. The miR-15a-miR-16-1 cluster controls prostate cancer by targeting multiple oncogenic activities. Nature Medicine. 2008;**14**(11):1271-127

[84] Hizir MS, et al. Simultaneous detection of circulating oncomiRs from body fluids for prostate cancer staging using nanographene oxide. ACS Applied Materials & Interfaces. 2014;**6**(17):14772-14778

[85] Samsonov R, et al. Lectin-induced agglutination method of urinary exosomes isolation followed by mi-RNA analysis: Application for prostate cancer diagnostic. Prostate. 2016;**76**(1):68-79

[86] Graves LE, et al. Proinvasive properties of ovarian cancer ascites-derived membrane vesicles. Cancer Research. 2004;**64**(19):7045-7049

[87] Taylor DD, Gercel-Taylor C. MicroRNA signatures of tumor-derived exosomes as diagnostic biomarkers of ovarian cancer. Gynecologic Oncology. 2008;**110**(1):13-21

[88] Rank A, et al. Circulating microparticles in patients with benign and malignant ovarian tumors. Anticancer Research. 2012;**32**(5):2009-2014

[89] Brock G, Castellanos-Rizaldos E, Hu L, Coticchia C, Skog J. Liquid biopsy for cancer screening, patient stratification and monitoring. Translational Cancer Research. 2015;**4**(3):280-290

[90] Kuhlmann JD, et al. microRNA and the pathogenesis of ovarian cancer--a new horizon for molecular diagnostics and treatment? Clinical Chemistry and Laboratory Medicine. 2012;**50**(4):601-615

[91] Larssen P, et al. Tracing cellular origin of human exosomes using multiplex proximity extension assay. Molecular & Cellular Proteomics. 2017;**16**(3):502-511

[92] Kahlert C, et al. Identification of double-stranded genomic DNA spanning all chromosomes with mutated KRAS and p53 DNA in the serum exosomes of patients with pancreatic cancer. Journal of Biological Chemistry. 2014;**289**(7):3869-3875

[93] Royo F, et al. Comparative miRNA analysis of urine extracellular vesicles isolated through five different methods. Cancers (Basel). 2016;**8**(12)

[94] Krafft C, et al. A specific spectral signature of serum and plasma-derived extracellular vesicles for cancer screening. Nanomedicine. 2017;**13**(3):835-841

[95] Cappello F, et al. Exosome levels in human body fluids: A tumor marker by themselves? European Journal of Pharmaceutical Sciences. 2017;**96**:93-98

[96] Zavesky L, et al. Evaluation of Cell-Free urine microRNAs expression for the use in diagnosis of ovarian and endometrial cancers. A pilot study. Pathology and Oncology Research. 2015;**21**(4):1027-1035

[97] Mitchell PJ, et al. Can urinary exosomes act as treatment response markers in prostate cancer? Journal of Translational Medicine. 2009;**7**:4

[98] Nilsson J, et al. Prostate cancer-derived urine exosomes: A novel approach to biomarkers for prostate cancer. British Journal of Cancer. 2009;**100**(10):1603-1607

[99] Motamedinia P, et al. Urine exosomes for Non-Invasive assessment of gene expression and mutations of prostate cancer. PLoS One. 2016;**11**(5):e0154507

[100] Royo F, et al. Transcriptomic profiling of urine extracellular vesicles reveals alterations of CDH3 in prostate cancer. Oncotarget. 2016;**7**(6):6835-6846

[101] Hendriks RJ, et al. Comparative analysis of prostate cancer specific biomarkers PCA3 and ERG in whole urine, urinary sediments and exosomes. Clinical Chemistry and Laboratory Medicine. 2016;**54**(3):483-492

[102] Huang X, et al. Exosomal miR-1290 and miR-375 as prognostic markers in castration-resistant prostate cancer. European Journal of Urology. 2015;**67**(1):33-41

[103] Ahadi A, et al. Long non-coding RNAs harboring miRNA seed regions are enriched in prostate cancer exosomes. Science Report. 2016;**6**:24922

[104] Li M, et al. An optimized procedure for exosome isolation and analysis using serum samples: Application to cancer biomarker discovery. Methods. 2015;**87**:26-30

[105] Drake RR, Kislinger T. The proteomics of prostate cancer exosomes. Expert Review of Proteomics. 2014;**11**(2):167-177

[106] Principe S, et al. In-depth proteomic analyses of exosomes isolated from expressed prostatic secretions in urine. Proteomics. 2013;**13**(10-11):1667-1671

[107] Dijkstra S, et al. Prostate cancer biomarker profiles in urinary sediments and exosomes. Journal of Urology. 2014;**191**(4):1132-1138

[108] Tanaka Y, et al. Clinical impact of serum exosomal microRNA-21 as a clinical biomarker in human esophageal squamous cell carcinoma. Cancer. 2013;**119**(6):1159-1167

[109] Wang H, et al. Expression of serum exosomal microRNA-21 in human hepatocellular carcinoma. Biomed Research International. 2014;**2014**:864894

[110] Liu J, et al. Increased exosomal microRNA-21 and microRNA-146a levels in the cervicovaginal lavage specimens of patients with cervical cancer. International Journal of Molecular Sciences. 2014;**15**(1):758-773

[111] Troyer DA, et al. A multicenter study shows PTEN deletion is strongly associated with seminal vesicle involvement and extracapsular extension in localized prostate cancer. Prostate. 2015;**75**(11):1206-1215

[112] Gabriel K, et al. Regulation of the tumor suppressor PTEN through exosomes: A diagnostic potential for prostate cancer. PLoS One. 2013;**8**(7):e70047

[113] Melo SA, et al. Glypican-1 identifies cancer exosomes and detects early pancreatic cancer. Nature. 2015;**523**(7559):177-182

[114] Consortium E-T, et al. EV-TRACK: Transparent reporting and centralizing knowledge in extracellular vesicle research. Nature Methods. 2017;**14**(3):228-232

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