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Extracellular vesicle isolation, purification and evaluation in cancer diagnosis

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Review

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Abstract

Strategies for non-invasive biomarker discovery in early detection of cancer are an urgent need. Extracellular vesicles (EVs) have generated increasing attention from the scientific community and are under intensive investigations due to their unique biological profiles and their non-invasive nature. EVs are membrane-enclosed vesicles with variable sizes and function. Such vesicles are actively secreted from multiple cell types and are considered as key vehicles for inter-cellular communications and signalling. The stability and potential to easily cross biological barriers enable EVs for exerting durable effects on target cells. These along with easy access to such vesicles, the consistent secretion from tumour during all stages of tumorigenesis and their content providing a reservoir of molecules as well as mirroring the identity of the cell of origin are virtues that have made EVs appealing to be assessed in liquid biopsy approaches and for using as a promising resource of biomarkers in cancer diagnosis and therapy and monitoring targeted cancer therapy. Early detection of EVs will guide time-scheduled personalised therapy. Surveying reliable and sensitive methods for rapid isolation of EVs from biofluids, the purity of isolated vesicles and their molecular profiling and marker specification for clinical translation in patients with cancer are issues in the area and the hot topics of many recent studies. Here, the focus is over methods for EV isolation and stratification for digging more information about liquid biopsy-based diagnosis. Extending knowledge regarding EV-based strategies is a key to validate independent patient follow-up for cancer diagnosis at early stages and inspecting the efficacy of therapeutics.

Introduction

Management of some tumour types has become an urgent dilemma. Over-diagnosis along with issues related to the prediction of patient survival are challenges still that exist in the context of cancer (ex. prostate cancer) (Ref. 1). Circulating biomarkers can be assessed as a tool for early detection of cancer and reducing the rate of mortality (Ref. 2). Liquid biopsy includes three conventional approaches including circulating tumour DNA (ctDNA), circulating tumour cells (CTCs) and extracellular vesicles (EVs) (Ref. 3). Evaluation of CTCs and ctDNA provide useful knowledge about tumour evolution and the risk of tumour recurrence. Such approaches, however, have limited clinical utility due to high rates of false-positive outcomes and low rates of detection (Ref. 4). However, multimodal analysis of ctDNA, CTCs and EVs is applicable using machine learning algorithm. Multimodal liquid biopsy can be used as an approach to improve the accuracy of liquid biopsy assays and to overcome low specificity and sensitivity of existing liquid biopsies (Ref. 5).

EVs have emerged recently as cancer biomarkers (Ref. 6). EVs contain genetic information of the original cell, and they can be used as blood biomarkers and in horizontal gene transfer for cancer therapy (Ref. 7). EVs secreted from tumour mediate cell-to-cell communication between tumour and its environment help to know more about cancer-related processes, such as progression and metastasis (Ref. 8). The cargo carried by EVs is transferred from donor to recipient or acceptor cells. The EV-based cell-to-cell communication can be mediated through activation of receptors on surface of recipient cells. EVs can also affect the phenotype of acceptor cell/s through transferring membrane encapsulating cargoes (Ref. 9). Characterisation of individual EVs and their subpopulations will expand the current knowledge about communications among cells in physiological and pathological conditions (Ref. 10). Generally, current approaches for EV purification demand time-consuming procedures. This along with high-cost antibodies and large sample volumes are predicaments for clinical utility of EVs in cancer diagnosis. In addition, there is no blood test specific for EV analysis in different types of cancers (Ref. 11). In this review, we aimed to discuss about a possibility of using EVs as a diagnostic biomarker in solid tumours. Here, we have a focus over different types of methods for EV isolation and specification along with novel EV-related biomarkers for early cancer diagnosis.

Extracellular vesicles in cancer

Over the past decade (Ref. 12), tumour-derived EVs are regarded as biomarker reservoirs for cancer diagnosis (Refs 3, 13). EV are secreted abundantly from many cell types into circulation

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(Ref. 4) and are able to easily cross biological barriers (Ref. 14). Human tumour microenvironment (TME) and plasma contain millions of diverse vesicles (Ref. 15). EV cargo is transferred both locally and systemically and directs interactions within the TME in favour of tumour aggression and metastasis (Ref. 16). Malignant tumour cells release EVs that their uptake by less malignant cells will boost metastatic capacity in such cells (Ref. 17). EV release from cancer cells is considerably higher compared with non-cancerous cells (Ref. 18). The number of EVs in plasma of healthy individuals is about 10^9–10^{10}/ml, but the concentration of such vesicles is increased to about 4- to 20-fold in disease conditions like cancer (Ref. 19). Over 10^4 EVs can be released from a single tumour cell per day, which makes tumour-derived EVs highly frequent compared with other biomarkers within circulation (Ref. 20). This is indicative of a need for developing sensitive methods for purification of EVs and their molecular profiling for clinical translation in cancer patients (Ref. 11). Recipient cells release EV cargo in pH ^{low} condition, and exchange of such vesicles will impact their phenotype (Ref. 9).

EVs are assessed for deciphering the underlying inflammatory pathways occurring due to psychoneurological symptoms in prostate cancer patients (Ref. 21). EVs harvested from pancreatic cancer patients are evaluated for protein cargo and comparting with that collected from healthy pancreatic epithelial cells. EVs from cancer cells were enriched in proteins responsible for vesicle generation and release along with oncogenic transformation of cells, whereas EVs from normal cells were enriched in proteins responsible for immune response. This indicates that tumour-derived EVs are able to initiate transformation of healthy cells into malignancy, and promote cancer spreading to other organs (Ref. 22). Communication between prostate cancer cells with bone environment for further progression of cancer toward bone metastasis is mediated by EVs. EVs are, in fact, working as vehicles for delivery of RNAs that increase the risk of metastasis and enabling the growth of tumour cells within bone (Ref. 8). EVs are also reported to be contributed to the metabolic events in breast cancer (Ref. 23), and that such vesicles are reprogrammed (through targeting IL-3Rα) to blunt metastasis of triple-negative breast cancer (TNBC) (Ref. 24). Purification of EVs from plasma in patients with ovarian, bladder and pancreatic cancer for surveying early cancer detection has shown the higher rate of early detection in pancreatic cancer compared with other cancer types (Ref. 25). This is indicative of the cancer-type dependence for EV-based stratification of patients at early-stage cancer.

Classification and characterisation of extracellular vesicles

EVs are classified into three subgroups including exosomes, microvesicles (MVs) and apoptotic bodies (Fig. 1). They have differences in biogenesis, composition and functions (Ref. 14). A recommended nomenclature for EV identification is based on their size, which is due to a difficulty for definition of such particles based on their sub-cellular origin (Ref. 26). MVs (microparticles or ectosomes (Ref. 27)) are large vesicles with a size ranging from 0.1 to over 1 μm (Ref. 28). The size of apoptotic bodies is reached to about 1–5 μm (Ref. 26). EVs are generally classified into small- and large-size subsets (Ref. 29), but they typically have a size lower than 100 nm (Ref. 28). Small EVs have a size lower than 200 nm (Ref. 26). Large EVs derived from cancer cells with a size ranged from 1 to 10 μm are called oncosomes (Ref. 30). Exosomes constitute a major part of small EVs (Ref. 27). Large EVs are mainly conferred to the MVs (Ref. 31). Large and small EVs are released from glioma cells. As compared with non-cancer human umbilical vein endothelial cells (HUVECs), large EVs are secreted at considerably higher number from glioma cells, but both cell sources release similar number of small EVs (Ref. 32).

Large vesicles or oncosomes isolated from plasma of prostate cancer patients are enriched in tumour DNA, and the DNA is reflective of the genetic aberrations in the cell-of-origin and the genomic make-up of cancer. The fraction of this chromosomal DNA is more abundant compared to that in small EVs in which the same patients showed negligible DNA amount in small EVs (Ref. 33). KRAS mutation is common in pancreatic ductal adenocarcinoma (PDAC), and is considered as a critical driver of tumorigenesis, contributed to the initiation of cancer and progression toward metastasis (Ref. 34). Evaluation of mutant *KRAS* DNA in pancreatic cancer showed a considerably higher amount in small EVs at early stage, while at late-stage cancer such mutant DNA is similarly distributed among small and large EVs (Ref. 35). Thus, DNA distribution among various EV sizes is different between tumours. EVs isolated from metastatic melanoma patients and assessment of associated proteins in different subpopulations showed the general enrichment of large EVs and evolving several markers that are believed to be unique for small EVs, but there are markers specific for either one. For instance, both large and small low-density EVs are enriched in flottilin-1, while ADAM10 is enriched exclusively in small low-density EVs, and mitofilin is enriched in large EVs (Ref. 36).

Exosomes are distinct from MVs both structurally and functionally (Ref. 37). Exosomes are formed by inward budding of plasma membrane in the form of multivesicular bodies (MVBs) and their cargo is assembled within such endocytic membranes, whereas ectosomes are formed by outward budding of plasma membrane and their cargo is accumulated within plasma membrane (Refs 38, 39) (Fig. 1). Recently, non-membranous nanoparticles with a size of about 35 nm called exomeres are identified (Ref. 40). The term EVP is used collectively for EVs and particles including exosomes and exomeres. EVPs are released at high concentrations (> 10^9) into peripheral blood, thereby providing adequate material for further analysis (Ref. 41). Liquid biopsies can be provided for proteomic profiling of EVPs, which is important for early detection of cancers and monitoring therapy responses (Ref. 15). Proteomic analysis of circulatory EVs *in vitro* can also be an effective tool for predicting their tissue-of-origin. In fact, proteomic profiling of EVs represents markers common among various cell types along with markers unique for a cell type. Diverse levels of the classical markers of small EVs, namely CD9, CD63 and CD81 are presented in different cell types (Ref. 42), but Grp78 and ARF6 are proteins related to apoptotic bodies and MVs, respectively (Ref. 43). Proteomic profiling of EVs from tissue explants and plasma in cancer patients showed 90% sensitivity for VCAN, THBS2 and TNC in tissue explant EVPs, and the sensitivity for plasma EVPs was 95% (Ref. 41). Immunoglobulins are the most frequent protein family packed within EVPs, and the specific types of EVP immunoglobulins can be used as a biomarker for distinguishing cancer patients from healthy individuals (Ref. 44).

Virtues for the use of extracellular vesicles in cancer diagnosis

EV isolation can be a useful tool for cancer diagnosis and therapy. Evaluation of body fluids is a non-invasive way for cancer diagnosis (Ref. 45), enabling detection of tumour risk in cancers hardly detected by routine methods. EV isolation from tumours like pancreatic cancer, which show cold immunity and represent poor prognosis (Ref. 46), can give us information about genetic changes and gene expression profile of this cancer type (Ref. 45). EV signature is able to distinguish metastatic or non-metastatic breast cancer with high accuracy (~ 91%) (Ref. 20). Isolation of EVs may provide important information for detection

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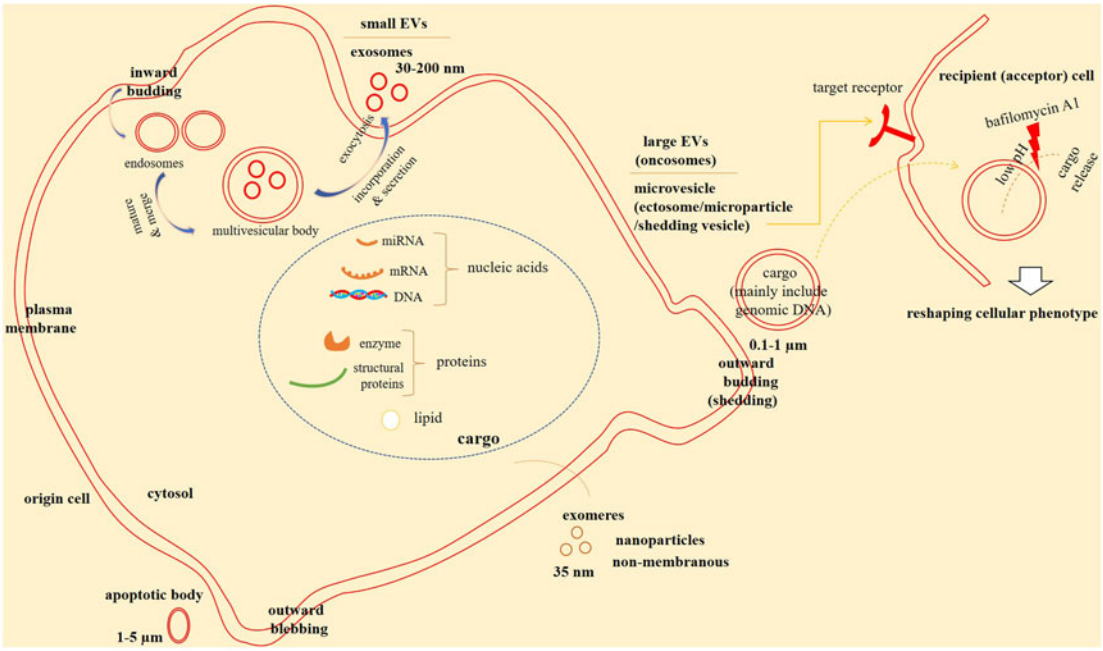


Fig. 1. Extracellular vesicles (EVs) in cell-to-cell communications. EVs are bilayer membrane structures that carry cargo including lipids, proteins and nucleic acids to affect target cells, acting as cell-to-cell communicators. Microvesicels (MVs), apoptotic bodies and exosomes are three subclasses of exosomes. Exosomes are generated from inward budding of plasma membrane in the form of endosomes. Endosomes are then become mature and merge to form mulivesicular bodies (MVBs) that are subsequently join the plasma membrane to release exosomes into the extracellular milieu. MVs are generated from outward budding of plasma membrane. Exomeres are non-membranous structures that are recently identified. Small EVs are mainly confer to the exosomes, while large EVs (also called oncosomes) are referred mainly to the MVs. EVs in recipient (or acceptor) cells release their cargo in pH ^{low} conditions and reshape the phenotype of such cells. Targeting EV cargo release in acceptor cells by agents, such as bafilomycin A1 can define therapy against cancer.

of diseases related to brain, such as cancers involving brain tissue. This is due to that EVs are able to cross blood-brain barrier (BBB) and transfer neurodegenerative and inflammatory molecules into the brain area (Ref. 21). EVs can access tissues of whole body (Ref. 47). Gut microbiome, for instance, is reflective of overall brain activity through the involvement of gut-brain axis. The microbiome affects the susceptibility of body to cancer, in which its altered activity can be contributed to the carcinogenic activity in an organ like brain. Generally, microorganisms cannot cross the BBB, but the EVs released from them have such capacity due to their nanosized diameter. Metagenomic analysis of microbial EVs collected from serum or brain tissue will provide a rich source of data for detection of brain tumour risk with high accuracy (Ref. 48).

EVs are traceable within different biological fluids, such as saliva, urine, blood and breast milk, reflecting molecular fingerprint of the cell of origin. Such diverse sources infer a possibility of a molecular EV readout practically for all organs within the body (Ref. 18). Blood contains a huge number of non-vesicular materials, such as protein aggregates and free proteins (Ref. 49), which requires appropriate techniques for effective isolation of EVs and identification of EV proteins. EVs can be traced within circulation at nearly early stages, and they are detectable throughout all disease courses (Ref. 3). Thus, EVs can be obtained from blood samples collected for monitoring treatment responses. Conventional methods, by contrast, require several biopsies, which are not feasible due to demanding financial costs and put patients at higher risks (Ref. 4). Another virtue for use of EVs in cancer diagnosis and therapy is their inherent stability (Ref. 3). This is due to the protection made by EV lipid bilayer, which is able to protect the EV cargo from further degradation by external enzymes and proteases (Refs 18, 50), thereby guarantying the integrity of cargos carried by them (Ref. 3). The cargo includes RNAs along with proteins that serve as fingerprint of the cell of origin (Ref. 51). This is indicative of the donor cell reflection of EV biochemical membrane composition, thereby granting the preferential tropism of exosomes toward their parented cell of origin (Ref. 52). The EV-based shuttle of miRNAs can be used as a prognostic and diagnostic biomarker in cancer patients. For instance, presence of miR-1246 in serum EVs from gallbladder cancer patients is indicative of cellular proliferation and invasion, and its combination with CA19-9 and CEA represents the higher diagnostic power in such patients. It is also reported that such miR type can be traced as an independent prognostic biomarker in such cancer (Ref. 53). Plasma profiling of EV-derived long RNAs is also reported to be of prognostic value in predicting treatment efficacy in breast cancer patients. Based on outcomes of one report, EV-derived long RNAs can be traced for distinguishing pathological responses in such cancer type (Ref. 54).

In-depth assessment of EV cargo yields higher specificity and sensitivity compared with whole plasma samples. This is due to that cancer-derived EVs are presumably enriched in molecules important for cancer diagnosis. These relevant molecules in such fraction are not detectable in plasma because of their mixing with highly frequent blood proteins (Ref. 18). In fact, EVs are enriched with contents that are selectively sorted from original cells. Furthermore, EVs are secreted actively from live cells rather than shedding from damaged or apoptotic cells (Ref. 55). EV cargo shows dynamic changes upon tumour progression (Ref. 56), which is indicative of the importance of EV evaluation for stage stratification.

Challenges for application of extracellular vesicles in cancer diagnosis

Like other agents, EV-based cancer therapy has its own issues, and current strategies are underway for solving such predicaments. First, isolation of purified EVs is a challenge in the area of EV-based therapy. EVs have rather small size, and

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contaminants are generally co-exist with these particles upon isolation (Ref. 29). Second, very low concentration of exosomes in serum samples (<10^9 vesicles/ml) is another issue (Ref. 45). Third, low levels of expressions for EV-related RNAs at early-stage cancer, detection of which at this stage is highly valuable (Ref. 57). Fourth, insufficient sensitivity, restricted throughput, extended time for sample workup and no validate biomarker are other burdens in regard with the use of EVs in clinical setting (Ref. 58). Fifth, heterogeneity of EVs will make difficulties their isolation and detection of their specific subpopulations from other non-target EVs within circulation (Ref. 6). Such heterogeneity is also referrable for some EV subtypes, such as exosomes in which two subpopulations of exosomes are identified: small (60–80 nm) and large (90–120 nm) exosomes (Ref. 59). Technical restrictions will be another issue, which hampers full separation of a specific subtype of EVs (Ref. 60).

Isolation of extracellular vesicles from tissue samples or plasma

Proteomic analysis of Extracellular vesicles and particles (EVP) harvested from human plasma and tissue samples gives us important information about the presence of tumour or even identifying tumour type. Based on results of a recent study, specificity of plasma and tissue EVP cargos for distinguishing tumour from normal tissues are 90 and 94%, respectively. Specific tumour-type proteins also exist within EVPs of plasma and tissue samples, which are useful biomarkers for detecting primary origin of tumour (Ref. 41). Pan-cancer markers are EVP adhesion molecules and some metabolic enzymes. Examples of adhesion molecules are tenascin C, versican, CD36 and thrombospondin 2. A point important for consideration is the high similarity in proteomic profiling of tissue or cell-line derived EVPs between human and murine, while EVPs harvested from plasma are vastly different between the two, indicating low reliability of results from murine plasma EVPs when interpreted to human. Another point is that some proteins are exclusively detectable in plasma, not in tissue explants, so EVP proteins are seemingly reflective of cancer-related systemic changes (Ref. 40). Whiteside recently quoted on the idea and announced that EVPs with unique cancer-related proteomic profile are traceable only from plasma of cancer patients (Ref. 15). EVs in plasma have a life-span of about 10 to 30 min, but they are constitutively released from cancer. It is impressive to note from a study by Lee *et* al.. who noticed a tumour suppressor activity for elevated plasm EVs after complete surgical removal of melanoma (Ref. 19). This is indicative of a possible change in the cargo presented by EVs and an impact of a surrounding milieu on such variation in the activity of EVs post-therapy.

Programmed death-ligand 1 (PD-L1) is a checkpoint that its overexpression in the TME represent poor prognosis and confers immunosuppression (Refs 61–64). In melanoma patients, circulatory exosomal PD-L1 is assessed in tissue biopsy and plasma samples. Exosomal PD-L1 is detected in plasma of all evaluated patients ($n = 100$), but only 67% of such cases were positive for PD-L1 in tumour biopsies. Comparisons are also made for soluble and exosomal PD-L1 within plasma, and there were considerably higher levels of this checkpoint in exosomes (64.26 pg/ml) compared with soluble PD-L1 (0.1 pg/ml). These are indicative that plasma detection of exosomal PD-L1 is more reliable than that detected in tumour biopsies, and quantification of PD-L1 is easier in exosomes compared to the soluble PD-L1 (Ref. 65). Serum assessment of exosomal PD-L1 is also applicable for monitoring metastasis in cancer patients, such as lung metastasis in osteosarcoma cases (Ref. 66). A key and basic tip here is that tissue biopsy is invasive by nature, whereas plasma allows us to have a non-invasive method. Considering this along with what aforementioned, it could be asserted that plasma evaluation of EVs can be a desired route for EV-based analysis for tumour genomic map in cancer patients.

Urine analysis of extracellular vesicles for detection of malignancies of urogenital system

Urine is ideal for evaluation of EVs in cancers of urological system, such as prostate cancer because of its close proximity with such system (Ref. 67). Prostasomes is an EV population detected in the prostatic and seminal fluid. Prostatic fluid is secreted into the urethra during urination, and its release can be stimulated using gentle prostate massage upon digital rectal examination (DRE), due to that the urine is called DRE urine. An increase in the amount of prostatic fluid in DRE urine is indicative of a rise in the rate of prostate-derived EVs (Ref. 68). Bottom-up OptiPrep density gradient (BU ODG) centrifugation for density-based urine fractionation is a useful tool for separation of highly-specific EVs from urine samples. In patients with prostate cancer, proteomic profiling of such vesicles can confirm the specificity of such cancer. BU ODG allows for gradient separation of three fractions of biomarkers: protein-enriched fraction at the bottom of ODG column (1.207–1.231 g/ml), Tamm-Horsfall Protein (THP)-enriched fraction (1.156–1.201 g/ml) overlaying the first and EV-enriched fractions on the top (1.087–1.109 g/ml) (Ref. 67). Urinary EVs can be traced for miRNAs in cancer detection and medical checkup. Detection of such EVs can serve as biomarkers for detection of urologic (ex. prostate and bladder) as well as non-urologic (ex. liver, lung and pancreas) cancers (Ref. 69).

Detection of extracellular vesicles

EVs carry tetraspanins and other CD markers on their surface like what seen on surface of stem cells. Such markers can be investigated and stratified into common and specific markers. Specific markers can be used for purification of EVs from plasma for further evaluation of their content in cancer diagnosis and therapy.

Tetraspanins for extracellular vesicle detection

Tetraspanins specific to the endosomes, such as CD9, CD63 and CD81 are enriched in human EVs (Ref. 70) and are considered as typical EV markers (Ref. 71). Expression of such tetraspanins along with the heat shock proteins (HSP70) can be served to distinguish EVs from other cytosolic vesicles (Ref. 72). CD9, CD63 and CD81 are all expressed in EVs derived from both prostate cancer stroma and the adjacent normal stroma with only low differences in their overall levels in EVs harvested from the two sources (Ref. 73). Anti-CD63 antibody can be used for capturing exosomes isolated by size-exclusion chromatography (SEC), as implemented for patients with head and neck cancer (Ref. 74). Magnetic beads and biochips can be conjugated with anti-CD9 antibody and used for capturing $CD9^+$ EVs from culture medium or plasma (Refs 75, 76). Based on outcomes of a recent report, an increase in the number of $CD63^+$ $CD81^+$ EVs in a tumour is interpreted as a better prognosis. High fraction of such EVs is accompanied by enriched pro-inflammatory phenotype of macrophages and increased cytolytic activity of immune system (Ref. 77).

Cancer-related CD markers

CDs 24, 29, 44 and 146 are cancer-related CD markers (Ref. 71). CD24 is a mucin-like antigen that is considered as an oncogene (Ref. 78) and a marker of cancer stemness (Ref. 79). CD24 is

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highly expressed in many types of solid cancers (Ref. 78), and $CD24^+$ EVs are detected in serum of patients with ovarian and breast cancers (Ref. 79). CD29 is implicated in cancer metastatic diffusion and is highly expressed in exosomes collected from cancer patients (Ref. 80). $CD44^+$ exosomal transfer from highly metastatic ovarian cancer cells will induce a metastatic phenotype in low-metastatic cancer cells (Ref. 81). CD44 is also described as a marker for detection of aggressive mesenchymal glioblastoma (GBM), which is identified at the surface of small EVs secreted from GBM cells. CD44 expression on small EVs from such cancer type is correlated with its mesenchymal phenotype and the invasive behaviour of cancer cells (Ref. 82). CD146 is an inducer of epithelial-mesenchymal transition (EMT), and is correlated with poor prognosis, high tumour stage and TNBC (Ref. 83). Reduced expression of CD146 in EVs at one month after surgical resection is indicative of the importance of this marker for disease monitoring after surgery and therapy (Ref. 84). CD29 along with CD146 are also enriched in EVs of patients with $Her2^+$ (*vs.* $Her2^-$) cancer (Ref. 71). In addition, CD146 is a well-known biomarker of melanoma that is also expressed by EVs derived from this cancer type (Ref. 85).

Extracellular vesicle contents for cancer detection

Melanoma signature using specific exosomal markers in human subjects is of prognostic and therapeutic value. This is comprised of HSP70, HSP90, TYRP2, VLA-4 and MET. Disease progression is predicted by exosomal co-expression of MET and TYRP2 (Ref. 86). Patients with malignant melanoma display P2X7 upregulation which is contributed to EV release and EV preferential miRNA selection, so it can be an appropriate prognostic marker and a therapeutic target in such patients (Ref. 87). PDCD6IP (ALIX) in small EVs is a protein that has the highest power for distinguishing patients with progressive melanoma from cases with no evidence of this disease. By contrast, upregulation of CNTN1 (contactin-1) occurs in small EVs only in cases with no evidence of melanoma following therapy (Ref. 88).

Exo-lncRNAs show considerable differences between breast cancer patients with healthy individuals. Among various exo-lncRNAs, VIM-AS1, ELDR and SNHG8 are found to be linked with disease progression (Ref. 89). Progressive colorectal cancer (CRC) cells release exosomal ADAM17 for their metastasis (Ref. 90). Exosomal Cripto-1 can be assessed in serum of patients with perihilar cholangiocarcinoma, and it can be considered as a potential biomarker (Ref. 91). Exosomal HOXD-AS1 is increased in serum of patients with prostatic cancer, and is regarded as a promising biomarker for diagnosis and treatment of the metastatic cancer. HOXD-AS1 is acting via modulation of the activity of miR-361-5p/FOXM1 axis, and is correlated with nodal and distant metastasis (Ref. 92). Post-DRE of exosomal lncRNA in urine is an effective way for early detection of prostatic cancer. Exploiting such way is superior to the routine prostate-specific antigen (PSA) testing, as it avoids unnecessary biopsies by 24.2% (Ref. 93). Epidermal growth factor receptor variant III (EGFRvIII) mRNA is identified in small and large EVs collected from glioma mice models (Ref. 32). In patients with GBM, MVs containing EGFRvIII are detected in serum of 7 per 25 cases (Ref. 94). EGFRvIII containing EVs are transferred from glioma cells toward $EGFRvIII^-$ cancer cells, which is for induction of the activation of related genes and pathways, thereby potentiating anchorage-independent growth of tumour (Ref. 95). A point to consider is that markers suggested here are not all specific for each type of cancer. Cripto-1, for instance, is expressed at high levels in several number of human cancers (Ref. 91). However, its evaluation can be an indicative of poor prognosis, and it can be assessed as a promising marker for early cancer detection. Thus, detection of Cripto-1 enables early management of cancer, thereby reducing the possibility of tumour metastasis. A summary of EV markers for detection of solid cancer types is presented in Figure 2.

Extracellular vesicle RNAs in solid cancer diagnosis

EVs mediate a dynamic and multi-directional communication between cells partly via delivering functional RNA (miRNA and mRNA) (Refs 96, 97). Cancer-derived exosomes are contributed to the biogenesis of miRNAs independent on cell involvement. Precursor miRNAs can thus be processed by cancer exosomes into mature miRNAs, and that such exosomes mediate rapid mRNA silencing for reprogramming the transcriptome of target cells. Such peculiarity for tumour-derived exosomes will offer potentials for developing exosome-based biomarkers (Ref. 98). miRNAs sorted to exosomes are seemingly modulated by the impact of cell activation on dynamic changes in transcriptome (Ref. 99), and distinct classes of EV RNAs are exported selectively by cells, evidence of which is in CRC (Ref. 16). Detection of EV-miR-1246 in serum is of prognostic and diagnostic value in patients with gallbladder cancer. miR-1246 promotes proliferation and invasion of gallbladder cancer cells and is considered as an independent prognostic biomarker (Ref. 53). Exosomal miR-25/miR-203 is a biomarker for detection and monitoring treatment responses in patients with oesophageal squamous cell carcinoma (ESCC). Exosomal miR-25/miR-203 level is increased in patients with ESCC, and it shows reduction in its level after surgery. It is also linked with the lymph node metastasis in such cases (Ref. 100). Compared with healthy subjects, urinary EV analysis from patients with benign prostatic hypertropia (BPH) and prostate cancer represents considerable upregulation of miR-21 and miR-346, while reduced levels of miR-23a and miR-122-5p were found in vesicles of such cases. Interestingly, levels of these miRNAs showed no considerable difference between patients with BPH and prostate cancer (Ref. 43).

Methods for detection of extracellular vesicle-associated RNAs in cancer patients: EV-associated mRNAs and miRNAs are biomarkers for detection of cancer (Ref. 57). Epigallocatechin-3-gallate (EGCG) is a polyphenolic biomolecule found in green tea, which is identified for its anti-oxidative activity and its anti-carcinogenic potential against a number of tumours (Ref. 101). EGCG can also be used for isolation and detection of exosomal miRNAs in human plasma samples. EVs can be efficiently isolated via this easy-to-use method, effective for detecting EV miRNAs in cancer patients. Polyphenol has potent adhesive tendency to biological molecules, which allows exosome collection within biofluids, such as whole blood, serum, plasma and urine (Ref. 45). Circulating EVs can be captured using nanoparticle biochips. Lipid-polymer hybrid nanoparticle (LPHN)-catalysed hairpin DNA circuit (CHDC) biochip is an effective tool for quantifying EV-associated RNAs. Signal amplification capacity allows the biochip to identify low levels of EV-mRNA in body fluids, such as serum, thereby enabling early detection of cancer. Low level of glypican-1 mRNA, for instance, can be detected using this biochip in serum EVs of patients with pancreatic cancer (Ref. 57).

ExoNA-sensing chip is a microfluidic device for accurate control of fluid flow and an effective tool for cancer diagnosis. This device is equipped with three-dimensional nanostructured hydrogels for one-step detection of specific RNAs in intact exosomes. In breast cancer, for instance, such chip is equipped with a sensing hydrogel with probes enabling detection of the target exosomal mRNA (the breast cancer marker *Erb-B2 receptor tyrosine kinase 2* [*ERBB2*] gene, along with a reference gene *GAPDH*). The hydrogel amplifies fluorescent signals at room temperature, and the encapsulation of probes with liposomes blocks generation of

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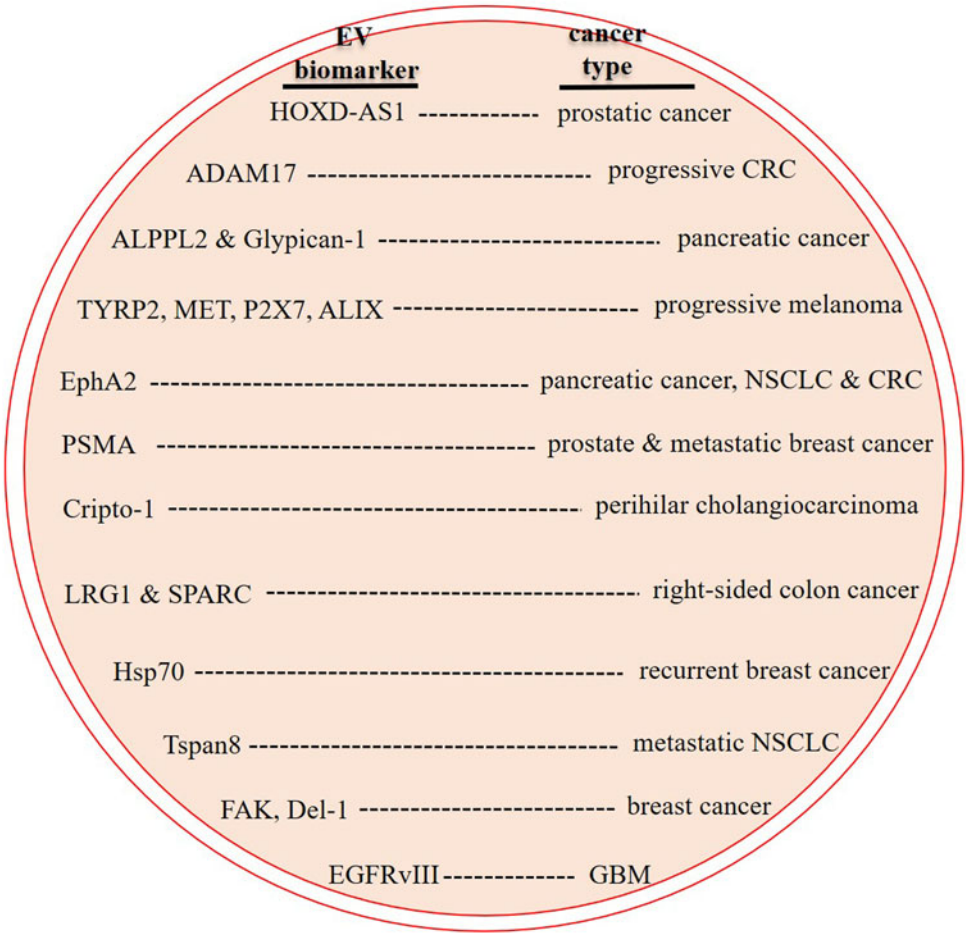


Fig. 2. Extracellular vesicle (EV) contents for cancer diagnosis. EphA2 and alkaline phosphatase placental-like 2 (ALPPL2) and glypican-1 are early diagnostic markers used for diagnosis of pancreatic cancer. Evaluation of EphA2 is also useful for detection of non-small cell lung cancer (NSCLC) and colorectal cancer (CRC). EVs from right-sided colon cancer are enriched in LRG1 and SPARC. Expression of etraspanin-8 on EVs can be a predictor of future metastasis in NSCLC patients. FAK enrichment of small EVs is a marker to distinguish breast cancer from healthy subjects. Surface expression of developmental endothelial locus-1 protein (Del-1) on EVs is a marker for early detection of breast cancer. Exosomal HOXD-AS1 can be assessed as a biomarker of metastatic prostate cancer, and prostate-specific membrane antigen (PSMA) is a biomarker of prostate cancer and metastatic breast cancer. An increase in the serum level of epidermal growth factor receptor variant III (EGFRvIII) within small EVs is seen in glioblastoma (GBM) patients. MET, TYRP2 and P2X7 are contributed to the progression of melanoma. P2X7 overexpression determines selective EV content release for metastatic spread. Cripto-1 assessment in exosomes can be a potential biomarker for patients with perihilar cholangiocarcinoma.

fluorescent signals from non-specific reactions. Precise analysis is also applicable via this device due to enabling the correction of value for variation in the number of exosomes among different individuals (Ref. 102).

Protocols for isolation and detection of extracellular vesicles

A number of methods are available for EV isolation and detection in cancer diagnosis, a summary of which is represented in Tables 1 and 2.

Labelling dyes

An ideal labelling method for visualisation of EVs requires to be specific for EVs, to has a desired half-life, to be stable and represent sufficient signal for preferential detection of nonoscaled EVs. Lipophilic dyes, such as PKH and DiI are commonly used for EV labelling (Ref. 96). Pre-incubation of exosomes with the PKH green dye and then exposing them to target cells, such as cancer cells will enable examination of exosomal internalisation in cell/s of target (Ref. 103). PKH, however, is not specific for EVs due to labelling other lipid-containing components as well. Live-cell imaging techniques can be obtained using florescent labelling of EV membrane. EV labelling using palmitoylated fluorescent protein (PalmtdTomato) dye is more specific than PKH67 (Ref. 96). PalmtdTomato is used for labelling membrane proteins on EVs (Ref. 104). PalmtdTomato labels predominantly the inner membrane of EVs, which will reduce potential disturbances in the composition of surface proteins in such vesicles (Ref. 96).

Enzyme-linked immunosorbent assay for extracellular vesicle quantification

Enzyme-linked immunosorbent assay (ELISA) is a method for EV quantification in unpurified liquid samples. Conventional EV ELISA has several predicaments, such as long assay time, large sample consumptions and limited sensitivity (Ref. 105). At least 50 μl undiluted plasma is required in a single replicate well of ELISA assay (Ref. 13). Conventional ELISA and western blot are based mainly on bulk (rather than precise) measurement of individual EV heterogeneity. Bulk measurements mask the differences exist among individual EVs. ExoELISA assay is an advanced method developed in this area for single EV evaluation. ExoELISA assay is able to count single exosomes within plasma (limit of

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Table 1. Common methods for extracellular vesicle (EV) isolation and purification in cancer

Method name	General description	Pros	Cons	Ref.
PKH staining	PKH is used commonly for labelling EV membrane lipids	Enabling examination of exosomal internalisation in cell/s of target	Not being specific due to also labelling other lipid-containing components	(Refs 96, 103)
ELISA	Conventional and ExoELISA are used for EV quantification in unpurified liquid samples	ExoELISA enables single EV count from plasma	Conventional method requires long assay time, large sample consumptions and limited sensitivity	(Refs 76, 105)
UC	UC is a commonly used method for EV isolation. Differential UC is proposed recently for EV isolation	Differential UC enables isolation of small & large EVs	UC is a time-consuming method for EV isolation	(Refs 20, 36)
SEC	SEC is a simple and reproducible way for isolation of highly pure EVs from blood samples	SEC enables rapid isolation of more functional and purer EVs due to better maintaining the integrity of small EVs, as compared with UC	Recovery of limited EV quantity	(Refs 108, 111)
precipitation	Precipitation is a commonly used method for EV isolation (ex, from urine)	A quite rapid method	Lower EV purity, as compared with SEC	(Ref. 111)

ELISA, enzyme-linked immunosorbent assay; UC, ultracentrifugation; and SEC, size-exclusion chromatography.

Table 2. Highly specific and novel methods for detection of **e**xtracellular vesicles (EVs) in cancer diagnosis

Method name	General description	Virtues	Ref.
PalmtdTomato	PalmtdTomato is used for labelling predominantly inner membrane proteins on EVs	More specific than PKH	(Ref. 96)
iddPCR	iddPCR is the latest utilisation of PCR technology	iddPCR detects rare proteins in a single EV with high sensitivity	(Refs 113, 114)
nPES	nPES is an ultrasensitive way which uses gold nanoparticles and a sensor chip for capturing tumour-derived EVs	nPES requires lower sample volume and it demands lower costs, as compared with ELISA. nPES yields rapid analysis with high specificity and sensitivity	(Ref. 13)
Thermophoretic analysis	Thermophoretic analysis is for tracing EV surface proteins and a promising way for early cancer diagnosis and classification	This method is an inexpensive and fast way for EV analysis with high sensitivity and specificity, and it requires low serum volumes	(Ref. 11)
LPHN-CHDC	LPHN-CHDC is a biochip for quantifying EV-associated RNAs	LPHN-CHDC enables detection of low levels of EV-mRNA in body fluids	(Ref. 57)
DPPIE	DPPIE is a method based on localised fluorescent signals generated from rolling circle amplification	DPPIE is an ultrasensitive tool for assessing individual EV heterogeneity via profiling multiple single EV proteins	(Ref. 76)
AFM-IR	AFM-IR is a spectroscopy-based method for identification of EV structure/composition	AFM-IR is able to detect proteins, nucleic acids and lipids within a single EV and comparing individual EVs both qualitatively and quantitatively	(Ref. 10)

iddPCR, immune-droplet digital PCR; nPES, nanoplasmon-enhanced scattering; LPHN-CHDC, Lipid-polymer hybrid nanoparticle-catalysed hairpin DNA circuit; DPPIE, digital profiling of proteins on individual EV; and AFM-IR, atomic force microscope IR spectroscopy.

detection (LOD): 10 exosomes/μl). This method can be used for distinguishing breast cancer cases before/after surgery (Ref. 76).

Ultracentrifugation

Differential ultracentrifugation (UC) followed by density separation is an approach recently proposed for EV isolation (Ref. 29). Generally, UC is a time-consuming method (Ref. 20) in which Conventional differential UC is a heavy workload due to requiring consecutive centrifugation steps (Ref. 106). Both small and large size EVs can be isolated using differential UC (Ref. 36). UC at 1 10 000 × g is exploited for collection of exosomes from CRC patients (Ref. 107).

A fraction of urinary EVs is removed along with larger THP matrices during early low-speed centrifugation of urinary specimens, which introduces a bias. Recovery of such trapped EVs is applicable by depolymerisation using dithiothreitol, but it will result in the generation of smaller THP fibres that can be a source of contamination due to their co-sedimentation with EVs separated during high-speed centrifugation steps. A suggested approach for effective recovery of THP-entrapped urinary EVs and reducing contamination with proteins is the incorporation of alkaline wash and THP polymer reduction (Ref. 106).

Size-exclusion chromatography

SEC is a simple and reproducible way for isolation of human EVs with high purity from blood samples (Ref. 53). Compared to the UC, SEC retain the integrity of small EVs and concomitantly reduces contamination with plasma proteins (Ref. 108). This is

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indicative of better recovery of urinary exosomal particles and proteins by SEC compared with UC (Ref. 109). Besides, it is shown that EVs isolated by SEC are more functional compared to that for UC. This point is of value when EVs are applied for therapy (Ref. 110). A disadvantage of SEC is the recovery of limited EV quantity (Ref. 111).

Precipitation

Differential UC, SEC and commercial precipitation methods, such as Exoquick are used commonly for separation of EVs from urine. Outcomes of a recent study, however, showed that such methods are sub-optimal for separation of highly-specific urine EVs (Ref. 67). Precipitation and SEC are rapid methods for EV isolation compared to the UC. PRotein Organic Solvent PRecipitation (PROSPR) and polyethylene glycol (PEG) are examples of precipitating agents for EV isolation. A predicament for using PEG in EV isolation is the co-precipitation of proteins and contaminants, while SEC yields clean EVs (Ref. 111). Based on outcomes of one study, precipitation method for EV isolation also co-precipitated 9–15% of plasma proteins (Ref. 112).

Immuno-PCR and immune-droplet digital PCR

Immuno-polymerase chain reaction (iPCR) is a powerful method for identification of EV proteins (Ref. 12). This immunoassay technique employs DNA for signal generation and utilises antigen–antibody interactions and amplification of PCR power. Immune-droplet digital PCR (iddPCR) is the latest utilisation of PCR technology. ddPCR can be integrated into iPCR for enabling protein detection with high sensitivity (Ref. 113). EV proteins are profiled using iddPCR amplification method, allowing multiplexed protein profiling of a single EV. EVs are labelled with antibody-DNA conjugates. Then, stochastic microfluidic EV is incorporated into droplets, and barcode signals are converted and amplified (using *in situ* PCR with florescent probes) for droplet imaging. The iddPCR enables ultrasensitive detection of rare proteins in a single EV. Microscopic resolution is not demanded for identification of single EV via such technique. This can be an effective tool for analysis of plasma EVs, enabling detection of molecular signature and monitoring therapy (Ref. 114).

Nanotechnology for extracellular vesicle detection

Nanoplasmon-enhanced scattering assay

Nanoplasmon-enhanced scattering (nPES) assay is an inexpensive and ultrasensitive way for quantification of tumour-derived EVs. Gold nanoparticles including nanospheres and nanorods are used in this assay. Here, a sensor chip is considered for capturing EVs using specific antibodies and bondage of antibody-conjugated gold nanoparticles to EVs on this sensor chip. This will generate a local plasmon effect for increasing the specificity and sensitivity of detection for tumour-derived EVs. In this approach, quantification is applicable with low plasma sample (1 μl). As compared to the ELISA method, nPES requires lower sample volume, and it demands lower costs. nPES EphA2-EV assay can be used for distinguishing pancreatic cancer from pancreatitis. It can also be applicable for tumour staging and inspecting early responses to therapy (Ref. 13). Nanoplasmonic exosome assay can be used for analysing exosomes secreted from ovarian cancer cells into ascites. Evaluation of CD24 and epithelial cell adhesion molecule (EpCAM) on ovarian cancer exosomes enables distinguishing them from benign cells (Ref. 115). CD24 and EpCAM are presented in exosomes of malignant ascites in patients with ovarian cancer (Ref. 116).

Templated nanoplasmonics is a technology for multiparametric analysis of exosomes directly from clinical biofields. The same vesicles are undergoing simultaneous biomolecular and biophysical assessments. Here, Specific assessment of exosomal biomarkers is achieved through *in situ* assembly and growth of gold nanoshells on such vesicles. Multi-selective evaluation of various exosome markers, such as miRNAs and proteins are applicable through inspecting optical signals received from quenching fluorescent probes from the target-bound vesicles. This will distinguish such vesicles from non-vesicle particles or free molecular targets. This technology yields a rapid analysis in which only 15 min is required for multiplexed assessment with just 1 μl of plasma sample (Ref. 117).

Nanopatterned microchips

Nanopatterned microchips are three-dimensional devices that enable analysis of the EV-related proteolytic enzymes through evaluation of their expression profile and proteolytic activity. Plasma samples can be obtained, and accurate classification of cancer patients is applicable by this approach. *In vitro* detection of cell invasion along with *in vivo* monitoring of tumour metastasis are applicable via this approach, which enables clinicians to be informed for taking personalised cancer therapy (Ref. 55).

Designing a microfluidic chip with three-dimensional nanopatterns enables detection of low exosomal levels in plasma and is important for early detection of cancer. In patients with ovarian cancer, such device can be exploited for detection of exosomal folate receptor alpha (FRα) protein, which can be a marker for early detection and monitoring progression of such cancer (Ref. 118). FRα is a receptor that is overexpressed in a number of solid cancers, such as TNBC, lung cancer and ovarian cancer, and upon bondage with folate it enables folate taken up by cells, which is a target for chemotherapy drugs, such as pemetrexed and methotrexate (Ref. 119).

Magnetic nanoparticles

Lactoferrin conjugated 2,2-*bis*(methylol)propionic acid dendrimer-modified magnetic nanoparticles (LF-*bis*-MPA-MNPs)-based approach is a rapid and simple method for EV isolation. LF-*bis*-MPA-MNPs are chimeric nanocomposites. LF-*bis*-MPA-M can be used for isolation of EVs from several biological samples. In LF-*bis*-MPA-M method, a combination of biorecognition, electrostatic interaction and physical absorption are exploited for EV isolation, so it can be a high-yield method for isolation of EVs from urinary samples in both laboratory and clinics (Ref. 43). Labelling MVs with magnetic nanoparticles and their detection by miniaturised nuclear magnetic resonance system yields a rapid and highly sensitive technique for direct profiling of circulating MVs. In a cancer like GBM, such system enables detection of specific MVs in cancer patients and allows comparison of the rate with that in non-cancer subjects. Such MVs can also be detected for predicting responses to therapy (Ref. 120). Magnetic nanoparticles can be used for assessment of EV glycans. Many biomarkers used for clinical diagnostic purposes are glycosylated. EVs are harbouring a rich glycome, which is reflective of disease progression. Integrated magnetic analysis of glycans in EVs (iMAGE) is a technology designed for transduction of EV glycans into magnetic signals by polycore magnetic nanoparticles. Such signals are quantified through a sensor, and the result is a rapid (lower than 30 min) and sensitive (detection of about 10^4 EVs) assay (Ref. 121).

ExoSCOPE

Real-time monitoring of targeted therapy provides important information about drug resistance in tumours and therapeutic

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responses (Ref. 6). ExoSCOPE is a nanotechnology platform that can be applied for such purpose. ExoSCOPE is used for measurement of EV drug dynamics via which drug-target engagement is evaluated simultaneous with changes in protein expression profiles in a specific EV subtype. Assessment of EV drug dynamics is reflective of drug occupancy within target cells, thereby enabling real-time monitoring of treatment potency for a target drug (Ref. 122).

Imaging systems for extracellular vesicle detection

Imaging flow cytometry (IFC) is proposed as a way for EV observation and quantification. Such technique requires low sample volume and reduced preparation time for specimens. IFC can be used for enhancing the sensitivity of particle detection for EV analysis (Ref. 123).

Multiplexed reporters are optical reporters for labelling multiple population of EVs, enabling tracking and visualisation of EVs secreted from tumour, along with their uptake and exchange between various cell types both *in vitro* and *in vivo*. Here, membranes of EVs with different sizes are labelled with PalmGFP, PalmtdTomato, and PalmtdTomato encoding transcripts are tagged with a MS2 RNA-binding sequence for further visualisation and monitoring of both EV membrane and EV-packaged mRNA cargo. Visualisation of such EVs within tumour tissue is applicable using multiphoton intravital microscopy (Ref. 96).

EV enrichment is occurring concurrent with a shift in metabolic systems toward biosynthesis, enabling *in situ* imaging of tumorigenic processes, including metastatic-related events in an unperturbed TME (Ref. 124). Metabolic switching is considered as a tumour hallmark (Refs 125, 126). Multicontrast nonlinear imaging can be utilised for visualising endogenous substances in breast cancer from both animal and human subjects. Fresh sections from human breast cancer can be readily imaged for detection of tumour-associated EVs. Nonlinear imaging can thus be a novel approach for cancer diagnosis and therapeutics (Ref. 124).

Extracellular vesicle-based early detection of cancer

Early detection of cancer is the most focus of current studies. For a cancer like prostate, a localised cancer has an excellent prognosis with 5-year overall survival (OS) of 100%, but upon metastasis this rate is reduced to about 30% (Ref. 14). Metastasis is, in fact, the cause of almost all deaths from prostate cancer (Ref. 127) and, in general, is contributed to more than 90% of deaths due to cancer (Ref. 128) . The 5-year OS rate for early-stage renal cancer and CRC is about 90%, but upon metastasis this rate is reduced to lower than 12% in the former and to lower than 10% in the latter (Ref. 14). These statistics are representative of the importance of early cancer detection and investigating novel biomarkers for such purpose in order to have effective cancer therapy. Cancer patients, particularly cases at early stages of disease, display low levels of biomarkers. Besides, detection of such low fractions may be impaired due to competition with non-specific peptides and proteins, which are abundant within blood. EVs due to being stable and able to keep their cargo contents, can be used for biomarker detection early in tumorigenesis (Ref. 13).

EphA2 is overexpressed on EVs collected from pancreatic cancer cells, but it is absent on EVs derived from normal pancreatic tissue. EphA2 overexpression also occurs in early-stage non-small cell lung cancer (NSCLC) and CRC, which is indicative of the essence of evaluation of this biomarker for early cancer detection (Ref. 13). Alkaline phosphatase placental-like 2 (ALPPL2) is a membrane protein and a potential biomarker for diagnosis of PDAC at early stages. ALPPL2 is available in EVs released from pancreatic cancer (Ref. 51). Glypican-1^+ circulatory exosomes is another biomarker for early detection of pancreatic cancer. Glypican-1^+ exosomes identify *KRAS* mutations, which has 100% correlation with the mutation of this gene in tumours. *KRAS* is a gene abundantly mutated in patients with pancreatic cancer (Ref. 129). Alternating current electrokinetics (ACE) platform for EV purification from plasma is able to detect pancreatic cancer at stage 1 of the disease in 95.5% of cases. This rate is higher compared with the respective 74.4 and 43.8% detection rate in ovarian and bladder cancer patients (Ref. 25).

Breast cancer is a leader of death in women and despite advances in the field, identifying novel choices for early detection of such cancer is a crucial challenge (Ref. 130). Diagnosis of breast cancer at early stages allows for immediate surgery without the need for prior radiation or chemotherapy, which represents an optimistic prognosis. Carcino-embryonic antigen (CEA) and carbohydrate antigen 15-3 (CA15-3) are biomarkers that are currently assessed for screening breast cancer and monitoring treatment responses. However, the specificity and sensitivity of such blood markers are low, which asks for surveying novel efficient diagnostic methods for early detection of breast cancer (Ref. 54). The sensitivity of CA15-3, which is the most frequently used marker for patients with metastatic breast cancer is about 60–70%. Developmental endothelial locus-1 protein (Del-1) is a surface protein on circulating EVs that its concentration is considerably higher in breast cancer patients, and its rate is returned to normal after surgical resection of tumour. The sensitivity and specificity of Del-1 for detection of breast cancer are 84–99% and 74–86%, respectively (Ref. 131). Extracellular vesicle long RNA (exLR) d-signature score is higher in breast cancer compared with a benign disease. The d-signature can be exploited for detection of early-stage breast cancer from the control and benign groups with area under the curve (AUC) of 0.94 and 0.88, respectively, which is indicative of the potential of exLR d-signature for detection of early-stage breast cancer. exLRs, such as exMSMO1 can also be assessed in plasma for predicting responses to the neoadjuvant therapy. Silencing MSMO1 considerably augmented sensitivity of breast cancer cell lines to chemotherapy (Ref. 54).

Thermophoretic analysis of extracellular vesicle surface proteins for early detection of prostate cancer

Each year, more than a million of needle biopsies from prostate are performed in the USA mainly due to fluctuations or elevation of PSA, which prone patients to the increased risk of infectious complications (Ref. 132). In addition, not all prostate cancer cases show elevated level of PSA in which 30% of men reach a false negative rate with PSA lower that 4 ng/ml (Ref. 73). It is also not applicable to link a specific rate of PSA level in patients with metastatic prostate cancer (Ref. 133). Besides, tracing for PSA as a cancer screening marker can cause overdiagnosis and overtreatment. Thus, optimal replacement for PSA testing is imperative for strengthening detection rate and reducing unnecessary biopsies (Ref. 93). Tracing surface proteins from EVs using thermophoretic profiling is a promising way for early cancer diagnosis and classification (Ref. 11). A virtue for the use of thermophoretic aptasensor is no need for EV pre-isolation. Instead, thermophoretic enrichment and aptamer-bound EVs are used for producing an amplified fluorescent signal, the intensity of which is indicative of expression of serum EV surface proteins (Ref. 20). Sensitivity and specificity of this method for detection of stage 1 cancers are 95 and 100%, respectively. In regard with prostate cancer, such approach can be exploited efficiently for detection of PSAs and discriminating prostate cancer from benign prostate enlargement. Thermophoretic profiling is inexpensive and fast way and requires low serum volumes (lower than 1 μl) (Ref. 11). Thermophoretic profiling is also applicable for

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discrimination of metastatic from non-metastatic breast cancer and for monitoring treatment responses of the metastatic cancer (Ref. 20).

Extracellular vesicles for detection of some solid cancer types

Extracellular vesicle proteomic assay for detection of breast cancer subtypes

In regard with breast cancer, proteome profiling of EVs released from diverse cell lines can give us important information about different molecular subtypes. Different subtypes of breast cancer have specific molecular pathways and diverse biological processes. Different kinases and proteins are evolved in EVs, so evaluation of EVs can be exploited as a non-invasive tool for discriminating between various breast cancer subtypes. It was found EV enrichment essentially with factors contributed to metastasis in patients with TNBC. These factors were proteins related to angiogenesis, cell migration and extracellular matrix (ECM) organisation. Cellular proliferation, by contrast, is a hallmark of HER2 breast cancer subtype, which was elicited in EVs harvested from this tumour subtype. Proteins enriched in HER2-EVs act in cell-to-cell adhesion and are related to the ERBB2 signalling (Ref. 18).

Extracellular proteomic assay for detection of colorectal cancer

CRC is a tumour that shows high rates of prevalence and mortality (Ref. 14). EV protein profile is different in patients with right-sided compared with left-sided colon cancer. Right-sided colon cancer is more prone to develop metastasis. Leucine-rich alpha-2-glycoprotein 1 (LRG1) and secreted protein acidic and rich in cysteine (SPARC) are upregulated in EVs collected from right-sided colon cancer, thus they can be considered as markers of tumour sidedness (Ref. 134).

EV Click Chips for diagnosis of hepatocellular carcinoma

EV Click Chips is an EV purification system for detection of hepatocellular carcinoma (HCC). In this system, four approaches are used for EV purification: EV capturing, multi-marker cocktails of antibodies, nano-structured substrates and micro-fluidic chaotic mixers. Plasma samples are collected, and HCC specific mRNA markers are quantified using reverse transcriptase PCR system (Ref. 3).

Evaluation of extracellular vesicle heterogeneity

EVs released from different individual cells display considerable variations in their phenotypes and quantity. Such high heterogeneity may also occur in tumour-derived EVs (Ref. 76). In fact, tumours have heterogenous nature due to genetic variations among individual cells, and that such heterogeneity is also reflective in EVs (Ref. 17). Based on interpretations of a quite recent systematic review, the heterogeneity in EV samples can be explained partially by lack of appropriate methods for proper dissection of EV composition. It requires attention that the biological composition of EV membranes is very heterogenous, so differences are seen among individual cells (Ref. 135). Information collected from EV-to-EV heterogeneity can be helpful for developing new methods for cancer diagnosis. Localised fluorescent signals generated from rolling circle amplification (RCA) which is a useful tool for assessing individual EV heterogeneity. The whole method is called digital profiling of proteins on individual EV (DPPIE). Here, an anti-CD9 engineered biochip and multiple DNA aptamers are used for EV capture from plasma and specific label of surface membrane proteins on EVs, followed by RCA for generation of localised fluorescent signals, which can be observed with a confocal microscopy. DPPIE has an ultrasensitive EV analysis capacity enabling profiling of multiple proteins on a single EV. DPPIE can be used for distinguishing the EV in patients with lung squamous cell carcinoma from that in lung adenocarcinoma cases (Ref. 76). Atomic force microscope IR spectroscopy (AFM-IR) is a useful tool for identifying the structure and composition of EVs, so it can be applied in cancer diagnosis and therapy. This approach only requires a basic understanding of chemistry and spectroscopy, and data are completed at about 24 h. AFM-IR is able to detect proteins, nucleic acids and lipids within a single EV from a population of vesicles and to compare different population of EVs. This technique will enable comparing individual EVs both qualitatively and quantitatively. AFM-IR has a high resolution (<20 nm), which allows for interrogating variations in structure and composition in a single EV. Assessment of such variations is also important for tracing changes occur upon encapsulation of EVs with drug molecules used for drug carriers in cancer therapy. A point to consider is that AFM-IR is semi-quantitative due to the heterogeneity of vesicles (Ref. 10).

Conclusions and future perspectives

Discovering highly accurate and non-invasive biomarkers in cancer diagnosis is the main focus of the current area of cancer research. Over the past decade, several molecular signatures are developed, but they have no overlaps and are failed for validation in independent cohorts of patients (Ref. 1). EVs are under continuous evaluation for being used as prognostic and diagnostic biomarkers. EVs provide a window toward genetic state of individual cancers (Ref. 7). They show heterogeneity in tumours and conventional methods are based mainly on bulk measurements (Ref. 76). PalmtdTomato is an effective way for labelling EV membrane proteins, as compared to the PKH67. In regard with ELISA, ExoELISA is preferred over conventional methods due to enabling single exosome count. Compared to the ELISA, nPES needs lower sample volume, and it demands lower costs. Isolation of small and large size EVs is applicable using differential UC. As compared to the UC, more functional EVs can be isolated using SEC. Precipitation and SEC are rapid methods for EV isolation compared to the UC. SEC yields clean EVs, while contaminants exist in precipitation methods. Rare single EV proteins can be detected using iddPCR. LF-bis-MPA-M is a high-yield method and a rapid and simple way for EV isolation from urine. AFM-IR allows for interrogating variations in structure and composition of a single EV, but it is semiquantitative. A number of works are being exploited for finding EV markers specific for a tumour type and enabling cancer detection at early stages. Thermophoretic profiling is a fast and an inexpensive way, which requires low serum volumes and rendering high sensitivity and specificity for early cancer detection without a need for EV pre-isolation. Research is underway for evolving more specific markers in order to distinguish different subtypes of EVs and to overcome challenges related to the complexity of biofluids for sample preparation, as well as resolving technical variations for further analysis of samples (Ref. 136). High-throughput EV profiling is now applicable using stimuli-mediated systems. Here, stimuli-responsive copolymers are installed onto phospholipid bilayer of exosomes, which enables exclusive enrichment and purification of exosomes, and non-vesicle particles are not co-exist in samples. Development of such systems will definitely boost research regarding the EVs in basic and clinics, and can be a substitute for conventional methods, such as precipitation

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and UC in terms of purity, isolation yield and retained bioactivity (Ref. 137). Another advance in the field is the use of reference biological materials, such as recombinant EV (rEV), which has biophysical and biochemical traits of sample EVs. rEVs are stable and can be tailored to express a protein of interest, such as cancer cell-specific membrane protein, and they can be served as an internal control for data normalisation and evaluation of pre-analytical variables (Refs 136, 138). In the future, we will be notifying of more advances in the field through developing more systems of isolation with high reliability in isolation and purification of EVs and marker-specific stratification of such vesicles for cancer diagnosis at the earliest stages. Due to the growing number of cancers diagnosed in the world and the chronic inflammation remained due to the SARS-CoV-2 induced disease pandemic event the need for reliable non-invasive biomarkers will be more sensible.

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References

1. **Kim Y *et al.*** (2016) Targeted proteomics identifies liquid-biopsy signatures for extracapsular prostate cancer. *Nature Communications* **7**, 1–10.
2. **Trinidad CV *et al.*** (2020) Reducing ovarian cancer mortality through early detection: approaches using circulating biomarkers. *Cancer Prevention Research* **13**, 241–252.
3. **Sun N *et al.*** (2020) Purification of HCC-specific extracellular vesicles on nanosubstrates for early HCC detection by digital scoring. *Nature Communications* **11**, 1–12.
4. **Liu Y *et al.*** (2020) Extracellular vesicle tetraspanin-8 level predicts distant metastasis in non–small cell lung cancer after concurrent chemoradiation. *Science advances* **6**, eaaz6162.
5. **Bu J *et al.*** (2021) Tri-modal liquid biopsy: combinational analysis of circulating tumor cells, exosomes, and cell-free DNA using machine learning algorithm. *Clinical and Translational Medicine* **11**. doi: 10.1002/ctm2.499.
6. **Wang J *et al.*** (2020) Tracking extracellular vesicle phenotypic changes enables treatment monitoring in melanoma. *Science Advances* **6**, eaax3223.
7. **Balaj L *et al.*** (2011) Tumour microvesicles contain retrotransposon elements and amplified oncogene sequences. *Nature Communications* **2**, 1–9.
8. **Probert C *et al.*** (2019) Communication of prostate cancer cells with bone cells via extracellular vesicle RNA; a potential mechanism of metastasis. *Oncogene* **38**, 1751–1763.
9. **Bonsergent E *et al.*** (2021) Quantitative characterization of extracellular vesicle uptake and content delivery within mammalian cells. *Nature Communications* **12**, 1–11.
10. **Kim SY *et al.*** (2019) High-fidelity probing of the structure and heterogeneity of extracellular vesicles by resonance-enhanced atomic force microscopy infrared spectroscopy. *Nature Protocols* **14**, 576–593.
11. **Liu C *et al.*** (2019) Low-cost thermophoretic profiling of extracellular-vesicle surface proteins for the early detection and classification of cancers. *Nature Biomedical Engineering* **3**, 183–193.
12. **Sun L and Meckes DG** (2021) Multiplex protein profiling method for extracellular vesicle protein detection. *Scientific Reports* **11**, 1–13.
13. **Liang K *et al.*** (2017) Nanoplasmonic quantification of tumour-derived extracellular vesicles in plasma microsamples for diagnosis and treatment monitoring. *Nature Biomedical Engineering* **1**, 1–11.
14. **Najafi M *et al.*** (2021) The current knowledge concerning solid cancer and therapy. *Journal of Biochemical and Molecular Toxicology* **35**, e22900.
15. **Whiteside TL** (2020) Validation of plasma-derived small extracellular vesicles as cancer biomarkers. *Nature Reviews Clinical Oncology* **17**, 719–720.
16. **Hinger SA *et al.*** (2018) Diverse long RNAs are differentially sorted into extracellular vesicles secreted by colorectal cancer cells. *Cell Reports* **25**, 715–725, e4.
17. **Zomer A *et al.*** (2015) *In vivo* imaging reveals extracellular vesicle-mediated phenocopying of metastatic behavior. *Cell* **161**, 1046–1057.
18. **Rontogianni S *et al.*** (2019) Proteomic profiling of extracellular vesicles allows for human breast cancer subtyping. *Communications Biology* **2**, 1–13.
19. **Lee J-H *et al.*** (2020) Evidence for liver and peripheral immune cells secreting tumor-suppressive extracellular vesicles in melanoma patients. *EBioMedicine* **62**, 103119.
20. **Tian F *et al.*** (2021) Protein analysis of extracellular vesicles to monitor and predict therapeutic response in metastatic breast cancer. *Nature Communications* **12**, 1–13.
21. **Sass D *et al.*** (2021) Extracellular vesicle associated and soluble immune marker profiles of psychoneurological symptom clusters in men with prostate cancer: an exploratory study. *Translational Psychiatry* **11**, 1–8.
22. **Servage KA *et al.*** (2020) Proteomic profiling of small extracellular vesicles secreted by human pancreatic cancer cells implicated in cellular transformation. *Scientific Reports* **10**, 1–13.
23. **Chen F *et al.*** (2019) Extracellular vesicle-packaged HIF-1α-stabilizing lncRNA from tumour-associated macrophages regulates aerobic glycolysis of breast cancer cells. *Nature Cell Biology* **21**, 498–510.
24. **Lopatina T *et al.*** (2020) Targeting IL-3Rα on tumor-derived endothelial cells blunts metastatic spread of triple-negative breast cancer via extracellular vesicle reprogramming. *Oncogenesis* **9**, 1–14.
25. **Hinestrosa JP *et al.*** (2022) Early-stage multi-cancer detection using an extracellular vesicle protein-based blood test. *Communications Medicine* **2**, 1–9.
26. **Ko SY *et al.*** (2019) Cancer-derived small extracellular vesicles promote angiogenesis by heparin-bound, bevacizumab-insensitive VEGF, independent of vesicle uptake. *Communications Biology* **2**, 1–17.
27. **Kowal J *et al.*** (2016) Proteomic comparison defines novel markers to characterize heterogeneous populations of extracellular vesicle subtypes. *Proceedings of the National Academy of Sciences* **113**, E968–E977.
28. **Feng Q *et al.*** (2017) A class of extracellular vesicles from breast cancer cells activates VEGF receptors and tumour angiogenesis. *Nature Communications* **8**, 1–17.
29. **Crescitelli R, Lässer C and Lötvall J** (2021) Isolation and characterization of extracellular vesicle subpopulations from tissues. *Nature Protocols* **16**, 1548–1580.
30. **DeRita RM *et al.*** (2019) Tumor-derived extracellular vesicles require β1 integrins to promote anchorage-independent growth. *Iscience* **14**, 199–209.
31. **Zeng A *et al.*** (2020) Glioblastoma-derived extracellular vesicles facilitate transformation of astrocytes via reprogramming oncogenic metabolism. *Iscience* **23**, 101420.
32. **Yekula A *et al.*** (2020) Large and small extracellular vesicles released by glioma cells *in vitro* and *in vivo*. *Journal of Extracellular Vesicles* **9**, 1689784.
33. **Vagner T *et al.*** (2018) Large extracellular vesicles carry most of the tumour DNA circulating in prostate cancer patient plasma. *Journal of Extracellular Vesicles* **7**, 1505403.
34. **Kamerkar S *et al.*** (2017) Exosomes facilitate therapeutic targeting of oncogenic KRAS in pancreatic cancer. *Nature* **546**, 498–503.
35. **Hagey DW *et al.*** (2021) Extracellular vesicles are the primary source of blood-borne tumour-derived mutant KRAS DNA early in pancreatic cancer. *Journal of Extracellular Vesicles* **10**, e12142.
36. **Crescitelli R *et al.*** (2020) Subpopulations of extracellular vesicles from human metastatic melanoma tissue identified by quantitative proteomics after optimized isolation. *Journal of Extracellular Vesicles* **9**, 1722433.
37. **Kanada M *et al.*** (2015) Differential fates of biomolecules delivered to target cells via extracellular vesicles. *Proceedings of the National Academy of Sciences* **112**, E1433–E1442.
38. **Meldolesi J** (2018) Exosomes and ectosomes in intercellular communication. *Current Biology* **28**, R435–R444.

https://doi.org/10.1017/erm.2022.34 Published online by Cambridge University Press

39. **Fan SJ *et al.*** (2020) Glutamine deprivation alters the origin and function of cancer cell exosomes. *The EMBO Journal* **39**, e103009.
40. **Sandim V and Monteiro RQ** (2020) Extracellular vesicle fingerprinting: the next generation for cancer diagnosis? *Signal Transduction and Targeted Therapy* **5**, 1–3.
41. **Hoshino A *et al.*** (2020) Extracellular vesicle and particle biomarkers define multiple human cancers. *Cell* **182**, 1044–1061, e18.
42. **Garcia-Martin R *et al.*** (2022) Tissue differences in the exosomal/small extracellular vesicle proteome and their potential as indicators of altered tissue metabolism. *Cell Reports* **38**, 110277.
43. **Dao TNT *et al.*** (2022) Chimeric nanocomposites for the rapid and simple isolation of urinary extracellular vesicles. *Journal of Extracellular Vesicles* **11**, e12195.
44. **Pelissier Vatter FA *et al.*** (2021) Extracellular vesicle–and particle-mediated communication shapes innate and adaptive immune responses. *Journal of Experimental Medicine* **218**, e20202579.
45. **Jang M *et al.*** (2019) Extracellular vesicle (EV)-polyphenol nanoaggregates for microRNA-based cancer diagnosis. *NPG Asia Materials* **11**, 1–10.
46. **Mortezaee K** (2021) Enriched cancer stem cells, dense stroma, and cold immunity: interrelated events in pancreatic cancer. *Journal of Biochemical and Molecular Toxicology* **35**, e22708.
47. **Wang S *et al.*** (2019) Proteomic analysis of urinary extracellular vesicles reveal biomarkers for neurologic disease. *EBioMedicine* **45**, 351–361.
48. **Yang J *et al.*** (2020) Brain tumor diagnostic model and dietary effect based on extracellular vesicle microbiome data in serum. *Experimental & Molecular Medicine* **52**, 1602–1613.
49. **Muraoka S *et al.*** (2022) Comprehensive proteomic profiling of plasma and serum phosphatidylserine-positive extracellular vesicles reveals tissue-specific proteins. *iScience* **25**, 104012.
50. **Chen I-H *et al.*** (2017) Phosphoproteins in extracellular vesicles as candidate markers for breast cancer. *Proceedings of the National Academy of Sciences* **114**, 3175–3180.
51. **Shin H-S *et al.*** (2019) Alppl2 is a potential diagnostic biomarker for pancreatic cancer-derived extracellular vesicles. *Molecular Therapy-Methods & Clinical Development* **15**, 204–210.
52. **Sancho-Albero M *et al.*** (2019) Cancer-derived exosomes loaded with ultrathin palladium nanosheets for targeted bioorthogonal catalysis. *Nature Catalysis* **2**, 864–872.
53. **Ueta E *et al.*** (2021) Extracellular vesicle-shuttled miRNAs as a diagnostic and prognostic biomarker and their potential roles in gallbladder cancer patients. *Scientific Reports* **11**, 1–13.
54. **Su Y *et al.*** (2021) Plasma extracellular vesicle long RNA profiles in the diagnosis and prediction of treatment response for breast cancer. *NPJ Breast Cancer* **7**, 1–10.
55. **Zhang P *et al.*** (2020) Molecular and functional extracellular vesicle analysis using nanopatterned microchips monitors tumor progression and metastasis. *Science Translational Medicine* **12**. doi: 10.1126/scitranslmed.aaz2878.
56. **Wendler F, Stamp GW and Giamas G** (2016) Tumor–stromal cell communication: small vesicles signal big changes. *Trends in Cancer* **2**, 326–329.
57. **Hu J *et al.*** (2017) A signal-amplifiable biochip quantifies extracellular vesicle-associated RNAs for early cancer detection. *Nature Communications* **8**, 1–11.
58. **Park J *et al.*** (2021) An integrated magneto-electrochemical device for the rapid profiling of tumour extracellular vesicles from blood plasma. *Nature Biomedical Engineering* **5**, 678–689.
59. **Zhang H *et al.*** (2018) Identification of distinct nanoparticles and subsets of extracellular vesicles by asymmetric flow field-flow fractionation. *Nature Cell Biology* **20**, 332–343.
60. **Zhang Y *et al.*** (2021) The effect of extracellular vesicles on the regulation of mitochondria under hypoxia. *Cell Death & Disease* **12**, 1–14.
61. **Mortezaee K and Majidpoor J** (2022) Im) maturity in tumor ecosystem. *Frontiers in Oncology* **11**, 813897.
62. **Mortezaee K and Majidpoor J** (2021) Key promoters of tumor hallmarks. *International Journal of Clinical Oncology* **27**, 1–14.
63. **Mortezaee K and Majidpoor J** (2021) The impact of hypoxia on immune state in cancer. *Life Sciences* **286**, 120057.
64. **Majidpoor J and Mortezaee K** (2021) Angiogenesis as a hallmark of solid tumors-clinical perspectives. *Cellular Oncology* **44**, 1–23.
65. **Cordonnier M *et al.*** (2020) Tracking the evolution of circulating exosomal-PD-L1 to monitor melanoma patients. *Journal of Extracellular Vesicles* **9**, 1710899.
66. **Wang J *et al.*** (2020) Exosomal PD-L1 and N-cadherin predict pulmonary metastasis progression for osteosarcoma patients. *Journal of Nanobiotechnology* **18**, 1–23.
67. **Dhondt B *et al.*** (2020) Unravelling the proteomic landscape of extracellular vesicles in prostate cancer by density-based fractionation of urine. *Journal of Extracellular Vesicles* **9**, 1736935.
68. **Ramirez-Garrastacho M *et al.*** (2021) Extracellular vesicles as a source of prostate cancer biomarkers in liquid biopsies: a decade of research. *British Journal of Cancer* **126**, 1–20.
69. **Yasui T *et al.*** (2017) Unveiling massive numbers of cancer-related urinary-microRNA candidates via nanowires. *Science Advances* **3**, e1701133.
70. **Jung J-H *et al.*** (2021) Dual size-exclusion chromatography for efficient isolation of extracellular vesicles from bone marrow derived human plasma. *Scientific Reports* **11**, 1–9.
71. **Ekström K *et al.*** (2022) Characterization of surface markers on extracellular vesicles isolated from lymphatic exudate from patients with breast cancer. *BMC Cancer* **22**, 1–17.
72. **Tucci M *et al.*** (2018) Serum exosomes as predictors of clinical response to ipilimumab in metastatic melanoma. *Oncoimmunology* **7**, e1387706.
73. **Shephard AP *et al.*** (2021) Stroma-derived extracellular vesicle mRNA signatures inform histological nature of prostate cancer. *Journal of Extracellular Vesicles* **10**, e12150.
74. **Theodoraki M-N *et al.*** (2018) Clinical significance of PD-L1 + exosomes in plasma of head and neck cancer patients. *Clinical Cancer Research* **24**, 896–905.
75. **Vonlaufen A *et al.*** (2001) Integrin $\alpha v\beta 3$ expression in colon carcinoma correlates with survival. *Modern Pathology* **14**, 1126–1132.
76. **Zhang J *et al.*** (2020) Localized fluorescent imaging of multiple proteins on individual extracellular vesicles using rolling circle amplification for cancer diagnosis. *Journal of Extracellular Vesicles* **10**, e12025.
77. **Fathi M *et al.*** (2021) Single-cell cloning of breast cancer cells secreting specific subsets of extracellular vesicles. *Cancers* **13**, 4397.
78. **Jing X *et al.*** (2018) CD24 Is a potential biomarker for prognosis in human breast carcinoma. *Cellular Physiology and Biochemistry* **48**, 111–119.
79. **Altevogt P *et al.*** (2021) Novel insights into the function of CD24: a driving force in cancer. *International Journal of Cancer* **148**, 546–559.
80. **Kang YT *et al.*** (2020) Dual-isolation and profiling of circulating tumor cells and cancer exosomes from blood samples with melanoma using immunoaffinity-based microfluidic interfaces. *Advanced Science* **7**, 2001581.
81. **Shen X *et al.*** (2021) Exosome-mediated transfer of CD44 from high-metastatic ovarian cancer cells promotes migration and invasion of low-metastatic ovarian cancer cells. *Journal of Ovarian Research* **14**, 1–11.
82. **Lane R *et al.*** (2019) Cell-derived extracellular vesicles can be used as a biomarker reservoir for glioblastoma tumor subtyping. *Communications Biology* **2**, 1–12.
83. **Zeng Q *et al.*** (2012) CD146, an epithelial-mesenchymal transition inducer, is associated with triple-negative breast cancer. *Proceedings of the National Academy of Sciences* **109**, 1127–1132.
84. **Bandini E *et al.*** (2021) Early detection and investigation of extracellular vesicles biomarkers in breast cancer. *Frontiers in Molecular Biosciences* **8**. doi: 10.3389/fmolb.2021.732900.
85. **Porcelli L *et al.*** (2021) uPAR + extracellular vesicles: a robust biomarker of resistance to checkpoint inhibitor immunotherapy in metastatic melanoma patients. *Journal for Immunotherapy of Cancer* **9**. doi: 10.1136/jitc-2021-002372.
86. **Peinado H *et al.*** (2012) Melanoma exosomes educate bone marrow progenitor cells toward a pro-metastatic phenotype through MET. *Nature Medicine* **18**, 883–891.
87. **Pegoraro A *et al.*** (2021) P2X7 promotes metastatic spreading and triggers release of miRNA-containing exosomes and microvesicles from melanoma cells. *Cell Death & Disease* **12**, 1–12.
88. **Pietrowska M *et al.*** (2021) Proteomic profile of melanoma cell-derived small extracellular vesicles in patients' plasma: a potential correlate of melanoma progression. *Journal of Extracellular Vesicles* **10**, e12063.
89. **Zhao X *et al.*** (2021) Analysis of the expression profile of serum exosomal lncRNA in breast cancer patients. *Annals of Translational Medicine* **9**. doi: 10.21037/atm-21-3483.
90. **Sun J *et al.*** (2021) Exosome-derived ADAM17 promotes liver metastasis in colorectal cancer. *Frontiers in Pharmacology* **12**. doi: 10.3389/fphar.2021.734351.

https://doi.org/10.1017/erm.2022.34 Published online by Cambridge University Press

91. **Hu C *et al.*** (2021) Exosomal cripto-1 serves as a potential biomarker for perihilar cholangiocarcinoma. *Frontiers in Oncology* **11**, 3144.
92. **Jiang Y *et al.*** (2021) Exosomal long noncoding RNA HOXD-AS1 promotes prostate cancer metastasis via miR-361-5p/FOXM1 axis. *Cell Death & Disease* **12**, 1–14.
93. **Li Y *et al.*** (2021) A novel urine exosomal lncRNA assay to improve the detection of prostate cancer at initial biopsy: a retrospective multicenter diagnostic feasibility study. *Cancers* **13**, 4075.
94. **Skog J *et al.*** (2008) Glioblastoma microvesicles transport RNA and proteins that promote tumour growth and provide diagnostic biomarkers. *Nature Cell Biology* **10**, 1470–1476.
95. **Al-Nedawi K *et al.*** (2008) Intercellular transfer of the oncogenic receptor EGFRvIII by microvesicles derived from tumour cells. *Nature Cell Biology* **10**, 619–624.
96. **Lai CP *et al.*** (2015) Visualization and tracking of tumour extracellular vesicle delivery and RNA translation using multiplexed reporters. *Nature Communications* **6**, 1–12.
97. **Valadi H *et al.*** (2007) Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nature Cell Biology* **9**, 654–659.
98. **Melo SA *et al.*** (2014) Cancer exosomes perform cell-independent microRNA biogenesis and promote tumorigenesis. *Cancer Cell* **26**, 707–721.
99. **Squadrito ML *et al.*** (2014) Endogenous RNAs modulate microRNA sorting to exosomes and transfer to acceptor cells. *Cell Reports* **8**, 1432–1446.
100. **Liu Z *et al.*** (2021) Exosome-mediated miR-25/miR-203 as a potential biomarker for esophageal squamous cell carcinoma: improving early diagnosis and revealing malignancy. *Translational Cancer Research* **10**, 5174–5182.
101. **Guo S *et al.*** (2005) Green tea polyphenol epigallocatechin-3 gallate (EGCG) affects gene expression of breast cancer cells transformed by the carcinogen 7, 12–dimethylbenz [a] anthracene. *The Journal of Nutrition* **135**, 2978S–2986S.
102. **Lim J *et al.*** (2022) Microfluidic device for one-step detection of breast cancer-derived exosomal mRNA in blood using signal-amplifiable 3D nanostructure. *Biosensors and Bioelectronics* **197**, 113753.
103. **Zhao H *et al.*** (2016) Tumor microenvironment derived exosomes pleiotropically modulate cancer cell metabolism. *Elife* **5**, e10250.
104. **Zaborowski MP *et al.*** (2019) Methods for systematic identification of membrane proteins for specific capture of cancer-derived extracellular vesicles. *Cell Reports* **27**, 255–268, e6.
105. **Tan X *et al.*** (2021) Quantification and immunoprofiling of bladder cancer cell-derived extracellular vesicles with microfluidic chemiluminescent ELISA. *Biosensors and Bioelectronics: X* **8**, 100066.
106. **Correll VL *et al.*** (2022) Optimization of small extracellular vesicle isolation from expressed prostatic secretions in urine for in-depth proteomic analysis. *Journal of Extracellular Vesicles* **11**, e12184.
107. **Dou R *et al.*** (2021) EMT-cancer cells-derived exosomal miR-27b-3p promotes circulating tumour cells-mediated metastasis by modulating vascular permeability in colorectal cancer. *Clinical and Translational Medicine* **11**, e595.
108. **Vinik Y *et al.*** (2020) Proteomic analysis of circulating extracellular vesicles identifies potential markers of breast cancer progression, recurrence, and response. *Science Advances* **6**, eaba5714.
109. **Guan S *et al.*** (2020) Characterization of urinary exosomes purified with size exclusion chromatography and ultracentrifugation. *Journal of Proteome Research* **19**, 2217–2225.
110. **Mol EA *et al.*** (2017) Higher functionality of extracellular vesicles isolated using size-exclusion chromatography compared to ultracentrifugation. *Nanomedicine: Nanotechnology, Biology and Medicine* **13**, 2061–2065.
111. **Gámez-Valero A *et al.*** (2016) Size-exclusion chromatography-based isolation minimally alters extracellular vesicles' characteristics compared to precipitating agents. *Scientific Reports* **6**, 1–9.
112. **Karttunen J *et al.*** (2019) Precipitation-based extracellular vesicle isolation from rat plasma co-precipitate vesicle-free microRNAs. *Journal of Extracellular Vesicles* **8**, 1555410.
113. **Zhou W** (2018) Development of immunomagnetic droplet-based digital immuno-PCR for the quantification of prostate specific antigen. *Analytical Methods* **10**, 3690–3695.
114. **Ko J *et al.*** (2020) Single extracellular vesicle protein analysis using immuno-droplet digital polymerase chain reaction amplification. *Advanced biosystems* **4**, 1900307.
115. **Im H *et al.*** (2014) Label-free detection and molecular profiling of exosomes with a nano-plasmonic sensor. *Nature Biotechnology* **32**, 490–495.
116. **Runz S *et al.*** (2007) Malignant ascites-derived exosomes of ovarian carcinoma patients contain CD24 and EpCAM. *Gynecologic Oncology* **107**, 563–571.
117. **Wu X *et al.*** (2020) Exosome-templated nanoplasmonics for multiparametric molecular profiling. *Science Advances* **6**, eaba2556.
118. **Zhang P *et al.*** (2019) Ultrasensitive detection of circulating exosomes with a 3D-nanopatterned microfluidic chip. *Nature Biomedical Engineering* **3**, 438–451.
119. **Scaranti M *et al.*** (2020) Exploiting the folate receptor α in oncology. *Nature Reviews Clinical Oncology* **17**, 349–359.
120. **Shao H *et al.*** (2012) Protein typing of circulating microvesicles allows real-time monitoring of glioblastoma therapy. *Nature Medicine* **18**, 1835–1840.
121. **Wang Z *et al.*** (2020) Dual-selective magnetic analysis of extracellular vesicle glycans. *Matter* **2**, 150–166.
122. **Pan S *et al.*** (2021) Extracellular vesicle drug occupancy enables real-time monitoring of targeted cancer therapy. *Nature Nanotechnology* **16**, 734–742.
123. **Clark RT** (2015) Imaging flow cytometry enhances particle detection sensitivity for extracellular vesicle analysis. *Nature Methods* **12**, i–ii.
124. **Tu H *et al.*** (2017) Concurrence of extracellular vesicle enrichment and metabolic switch visualized label-free in the tumor microenvironment. *Science Advances* **3**, e1600675.
125. **Mortezaee K** (2021) Redox tolerance and metabolic reprogramming in solid tumors. *Cell Biology International* **45**, 273–286.
126. **Mortezaee K and Majidpoor J** (2022) Dysregulated metabolism: a friend-to-foe skewer of macrophages. *International Reviews of Immunology*, 1–17.
127. **Majidpoor J and Mortezaee K** (2021) Steps in metastasis: an updated review. *Medical Oncology* **38**, 1–17.
128. **Mortezaee K** (2021) Organ tropism in solid tumor metastasis: an updated review. *Future Oncology* **17**, 1943–1961.
129. **Melo SA *et al.*** (2015) Glypican-1 identifies cancer exosomes and detects early pancreatic cancer. *Nature* **523**, 177–182.
130. **Esposito CL *et al.*** (2021) Identification of a novel RNA aptamer that selectively targets breast cancer exosomes. *Molecular Therapy-Nucleic Acids* **23**, 982–994.
131. **Moon P-G *et al.*** (2016) Identification of developmental endothelial locus-1 on circulating extracellular vesicles as a novel biomarker for early breast cancer detection. *Clinical Cancer Research* **22**, 1757–1766.
132. **McKiernan J *et al.*** (2018) A prospective adaptive utility trial to validate performance of a novel urine exosome gene expression assay to predict high-grade prostate cancer in patients with prostate-specific antigen 2–10 ng/ml at initial biopsy. *European Urology* **74**, 731–738.
133. **Rode MP *et al.*** (2021) miR-425-5p as an exosomal biomarker for metastatic prostate cancer. *Cellular Signalling* **87**, 110113.
134. **Zhong M-E *et al.*** (2019) Serum extracellular vesicles contain SPARC and LRG1 as biomarkers of colon cancer and differ by tumour primary location. *EBioMedicine* **50**, 211–223.
135. **Gudbergsson JM *et al.*** (2019) Systematic review of targeted extracellular vesicles for drug delivery–considerations on methodological and biological heterogeneity. *Journal of Controlled Release* **306**, 108–120.
136. **Geeurickx E *et al.*** (2019) The generation and use of recombinant extracellular vesicles as biological reference material. *Nature Communications* **10**, 1–12.
137. **Liu X *et al.*** (2021) Stimuli-mediated specific isolation of exosomes from blood plasma for high-throughput profiling of cancer biomarkers. *Small Methods* **6**, 2101234.
138. **Geeurickx E *et al.*** (2021) Recombinant extracellular vesicles as biological reference material for method development, data normalization and assessment of (pre-) analytical variables. *Nature Protocols* **16**, 603–633.

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