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Exosome levels in human body fluids: A tumor marker by themselves?



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ABSTRACT

Despite considerable research efforts, the finding of reliable tumor biomarkers remains challenging and unresolved. In recent years a novel diagnostic biomedical tool with high potential has been identified in extracellular nanovesicles or exosomes. They are released by the majority of the cells and contain detailed molecular information on the cell of origin including tumor hallmarks. Exosomes can be isolated from easy accessible body fluids, and most importantly, they can provide several biomarkers, with different levels of specificity. Recent clinical evidence shows that the levels of exosomes released into body fluids may themselves represent a predictive/diagnostic of tumors, discriminating cancer patients from healthy subjects. The aim of this review is to highlight these latest challenging findings to provide novel and groundbreaking ideas for successful tumor early diagnosis and follow-up.

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1. Introduction

Research performed over recent years has demonstrated that body fluids contain substantial amounts of extracellular vesicles (EV) with sizes ranging between 30 and 1000 nm, which are surrounded by phospholipid membranes characterized by different micro-domains such as lipid-rafts and caveolae. Two main types of EVs have been described, nanosized exosomes (30–150 nm), that are derivatives of the endosomal system, and microvesicles (100–1000 nm) that are produced by outward budding of the plasma membrane (Yañez-Mó et al., 2015).

Exosomes are released by most cell types and mediate targeted intercellular communication under physiological and pathophysiological conditions, including different types of cancer (Ludwig and Giebel, 2012). Fig. 1 shows representative TEM pictures of epon embedded samples of human lung mucoepidermoid cells and EVs isolated from the same cells. The variety in size of intracellular vesicles contained within multivesicular bodies is clear and the same variety is present on EVs purification while the ultracentrifugation repeated rounds reduce the vesicle diameter to a range in which exosomes are enclosed. However, also other ultrastructural parameters may help in distinguishing exosomes from the other EVs, such as the typical bilayer membrane structure (Fig. 1 B and C).

Since EVs contain cell-type specific signatures, they have been proposed as biomarkers in a variety of diseases (Colombo et al., 2012; Gamez-Valero et al., 2015). They are composed by specific sets of molecules including proteins, lipids, metabolites and nucleic acids that altogether reflect the source cell (Lo Cicero et al., 2015; Chevillet et al., 2014). According to their molecular signature they can reach selected target cells at local or distance sites, within or between organs (Cantaluppi et al., 2012; Quesenberry et al., 2015; Hoshino et al., 2015). Giving their features, EVs provide ideal candidates as potentially the most reliable biomarkers in medicine (Fais et al., 2016). The majority of the available clinical data supporting the use of EVs as a source of disease biomarkers have been obtained from studies in cohort of cancer patients. This, of course, might be of paramount importance for the clinical management of tumor patients, but probably for ensemble of the human diseases, being, unfortunately, the identification of specific disease biomarkers an unmet clinical need. However, the scientific community is producing a huge effort in trying to discover new EVs-related disease biomarkers (Fais et al., 2016), possibly useful as specific targets for new therapeutic approaches as well (Lener et al., 2015).

2. The Weakness of Existing Tumor Biomarkers and the Promising Role of Plasmatic Exosomes

Currently the diagnosis and the follow-up of cancer patients suffer the absence of specific markers. In fact, clinical oncologists very often

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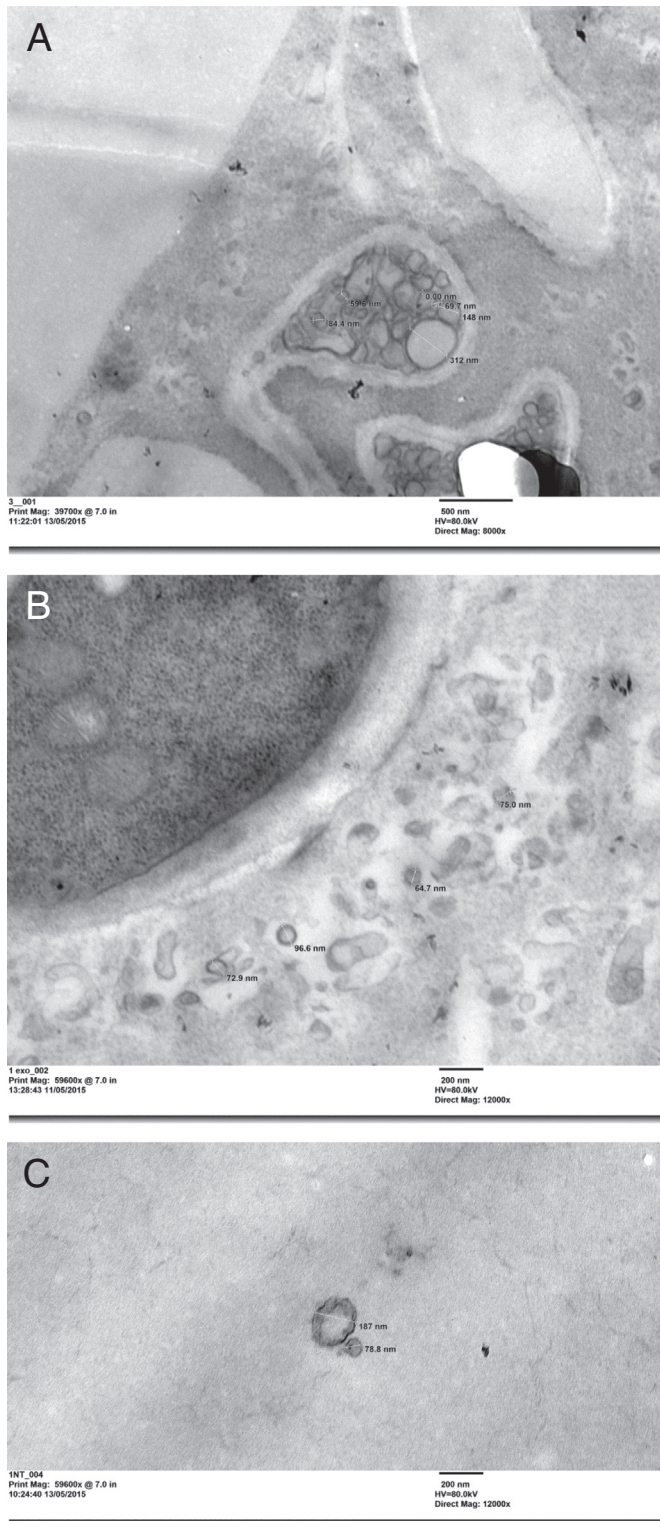


Fig. 1. Representative TEM images of epon-embedded and sectioned exosomes from cell cultures (NCI-H292 cells, human lung mucoepidermoid cells) supernatant.

use the available markers as a whole, and implement them by other non-specific markers to have a comprehensive general sight on the health status patient-by-patient. Plasmatic levels of existing tumor markers may change accordingly with the disease status, the side effects of current therapies and the co-existence of clinical complications. Moreover, too often tumor biomarkers may increase in other disease conditions with very low level of specificity, which does not allow

reliable screening test and too often leads to mis- or overdiagnosis. A clear example is one the most used tumor biomarkers, the prostatic specific antigen (PSA), which exploited worldwide as a prostate cancer (PCA) marker. In fact, serum PSA quantification is used in PCA screening from a couple of decades, with the idea to rapidly replace the digital rectal examination for early detection of prostate cancer (Catalona et al., 1991; Schröder, 2009; Brawley, 2012). Nevertheless, this diagnostic method is quite controversial (Andriole, 2009; Hoffman, 2011; Sturgeon et al., 2008) due to the likelihood of false positives, including benign prostatic hyperplasia (BPH) (Hoffman, 2011). Since PSA testing fails to discriminate between BPH and tumors, over-diagnosis and over-treatment are frequently a problem, increasing patient suffering due to the well known side effects of the few therapeutic options (Etzioni and Feuer, 2008; Schröder, 2009; Hoffman, 2011; Vickers et al., 2014). PSA values above 4.0 ng per milliliter are considered abnormal, however, cut-off levels can change with age, race and individual physiological conditions (Etzioni and Feuer, 2008; Schröder, 2009; Hoffman, 2011), and no significant progress has been achieved in the last decades (Steuber et al., 2008). Interestingly, some preliminary clinical studies have shown that PSA is detectable in preparations of plasma and urine-derived EVs (Mitchell et al., 2009; Mizutani et al., 2014). This suggests that studies aimed at quantification and characterization of PSA-associated plasmatic and urine nanovesicles may be extremely helpful in distinguishing between cancer and benign tumors, thus allowing screening test in extended males populations. This should be also true for all the existing clinical biomarkers of tumors, that in fact are not specific. Currently, available biomarkers for cancer screening and diagnosis regularly display a low level of specificity, while showing some sensitivity, not discriminating patients at early stages of disease (false negatives), or detecting those with no disease (false positives). Tumor-derived EVs are proposed to contain tumor-specific molecular signatures, qualifying them as potential biomarkers in tumor diagnostics (Zocco et al., 2014). EVs can be purified using sequential ultracentrifugation rounds from almost all body fluids, including blood and urine. As recently reported, the clinical evidence on the relevance of EVs as disease biomarkers is rapidly increasing (Fais et al., 2016). In fact, due to their complex assembly, exosomes may offer the potential of being more sensitive and specific than currently available clinical biomarkers (Mitchell et al., 2009). Real clinical studies are scarce, the majority describing the detection of novel markers in exosome preparations, but only few of them focus on the clinical relevance of the discovery. Therefore it is difficult to have a clear view on the issue, and the clinical data are so scattered that it is virtually impossible to propose them but with rambling summaries of the single reports. The result is that there are not yet clinical studies with a reliable evidence that plasmatic EVs bear markers of a disease, or, in the case of tumors, markers able of clear discrimination between different tumor histologies. It is out of the purpose of this review to summarize them, so here we just suggest the readers some recent position papers on this topic (Iero et al., 2008; Properzi et al., 2013; Properzi et al., 2013; Zocco et al., 2014; Fais et al., 2016).

Nevertheless, although a number of studies suggest that exosomes may contain specific markers useful for tumor early diagnosis and follow-up, these markers are still a sort of Holy Grail for cancerologists. Our hypothesis is that, “rather than to the finger one should look to the sky”: tumor patients have higher plasmatic exosome levels as compared to the controls. The ensemble of the clinical data on EVs levels in various body fluids of tumor patients to date does not support the existence of clear and specific biomarkers. However, some pre-clinical data actually suggest that exosomes may represent a preferential vehicle for biomarkers released by tumors. In this context, a translational study provided evidence of feasible human body fluids EVs quantification by an immunocapture-base ELISA test (Logozzi et al., 2009). This assay has shown that circulating exosome levels may reflect the tumor mass, using a surrogate tumor marker, such as caveolin, or simply a housekeeping exosome marker such as CD63; using both parameters tumor patients showed higher EVs marker as compared to healthy

volunteers (Logozzi et al., 2009). In the same paper, data relating the plasmatic exosome levels to the tumor size are shown (Logozzi et al., 2009). Some pre-clinical data suggest that the tumor microenvironmental conditions, such as extracellular acidity, may trigger an increased exosome release, that is inhibited through the use of various anti-acidic approaches, including both buffers and proton pump inhibitors (Parolini et al., 2009; Federici et al., 2014). In particular, tumor acidity is related to an increased tumor exosomes plasmatic levels (Federici et al., 2014). This suggests that the microenvironmental conditions, that are not present in the same tissues in both healthy or benign tumor condition, may favour the presence of tumor markers on exosomes, for instance of some enzymes such as PSA, needing an acidic microenvironment for a full activation. This may be the case of survivin as well, whose presence on plasmatic exosomes of prostate cancer patients has been proposed as an early detection marker of prostate cancer (Khan et al., 2012).

3. Are Plasmatic Exosome Levels a Marker of Tumor Progression and Recurrence?

The first evidence supporting the notion that EVs levels in human body fluids may represent potential markers for tumor progression was provided by a clinical study exploiting an immunocapture-based assay (Logozzi et al., 2009). The study showed that a considerable amount of melanoma patients with advanced disease presented significant higher level of plasmatic exosomes as compared to healthy volunteers. More in particular, stage III and IV melanoma patients showed increased levels of plasmatic caveolin-1 and CD63-positive EVs (Logozzi et al., 2009). EV-associated caveolin-1 displayed a sensitivity of 69% and specificity of 96.3%, while a conventional cancer biomarker used in the follow-up of melanoma patients, such as Lactate dehydrogenase (LDH) serum levels, was altered in only 12.5% of patients (Logozzi et al., 2009). These findings have received support by a recent study showing that in melanoma patients MIA and S100B positive exosomes were significantly higher than in healthy controls (Alegre et al., 2016). This hypothesis has been further suggested by another recent paper (Caivano et al., 2015), showing by FACS analysis that the amount of exosomes isolated from the serum of patients with different types of hematological tumours was significantly higher than in healthy subjects. Interestingly, they identified markers on circulating nanovesicles reflecting their cellular source, such as CD19 in B cell neoplasm, CD38 in multiple myeloma, CD13 in myeloid tumors and CD30 in Hodgkin's lymphoma (Caivano et al., 2015). According to these findings, a recent study has shown that in PCA patient nanovesicles levels are increased in body fluids, when measured by a time-resolved fluorescence immunoassay, capturing CD9 and CD63 positive exosomes (Duijvesz et al., 2015). A clinical study performed in patients with colorectal cancer supported these evidences obtained in other tumor histologies (Silva et al., 2012). The levels of plasmatic nanovesicles were quantified in 91 colorectal cancer patients and the results showed that they were statistically higher than in healthy controls. Moreover, exosome levels correlated with high levels of serum carcino-embryonic antigen (CEA). Further analysis showed that exosome levels significantly correlated with both, lower level of tumor differentiation and shorter overall survival (Silva et al., 2012). In prostate cancer patients the EV concentration, as measured by nanoparticle tracking analysis (NTA), was proven higher than in the plasma of healthy controls (Nawaz et al., 2014). Recently, a milestone study has shown that glypican-1 (GPC1) positive exosomes were detectable in the serum of patients with pancreatic cancer with high level of specificity and sensitivity, and could distinguish healthy subjects and patients with a benign pancreatic disease from patients with early- and late-stage pancreatic cancer (Melo et al., 2015). However, the same study showed that high levels of GPC1 on nanosized EVs were also detected in breast cancer patients, suggesting that an increase of circulating EV might represent a sign of the presence of a malignant cancers, suitable to be used in the clinical follow-up of cancer patients.

This may represent a valuable tool in cancer screening and in the assessment of the clinical status, as well.

Interesting data suggest that the levels of circulating EVs may be also exploited in assessing the effectiveness of a therapy in cancer patients. For instance, the effect of the treatment with Imatinib for gastrointestinal stromal tumor was monitored, showing that the concentration of EVs before the treatment was increased with respect to control (Ogorevc et al., 2013). Furthermore, in a preclinical setting, treatment with proton pump inhibitors in xenograft with human melanoma has shown that EVs plasmatic levels are consistent with reduction of the tumor size (Federici et al., 2014).

An interesting study has been very recently published, showing an increase in EVs release from irradiated compared to non-irradiated tumor cells (Mutschelknaus et al., 2016). The authors also show an enhanced uptake of EVs from both irradiated and non-irradiated cells by irradiated recipient cells compared to non-irradiated recipient cells, increasing survival. These results demonstrate that radiation influences both the abundance and action of exosomes on recipient cells and that exosomes transmit pro-survival effects by promoting the proliferation and radioresistance of head and neck cancer cells (Mutschelknaus et al., 2016). Circulating EVs have been included in the novel concept of "Liquid Biopsies". This is a significant acknowledgment to clinical results supporting the hypothesis that plasmatic levels of EVs may represent a valuable tumor marker (Logozzi et al., 2009; Silva et al., 2012; Alegre et al., 2016; Caivano et al., 2015; Duijvesz et al., 2015; Melo et al., 2015), and also to preclinical data showing that exosome levels correlate with the tumor mass (Logozzi et al., 2009). It is also suggested that the amount of circulating EVs may represent a new category, that we want here call for the first time "Liquid Tumor Mass". This notion needs further support from a larger number of clinical studies, correlating the circulating exosome levels to the whole tumor mass by mean of routine diagnostic examinations, including CT Scan, MNR and PET. However, "Liquid Tumor Mass" (LTM) wants also to include the concept that circulating EVs may actively contribute to the metastatic tumor dissemination, not only in preparing a sort of niche for the better seeding of circulating tumor cells into the target organs (Peinado et al., 2012), but also transforming local stem cells in a tumor-like manner (Lugini et al., 2016). Importantly, a general agreement on the method/s to quantify circulating EVs is required. Indeed, in all the studies reported in this review the EVs levels were quantified using quite distinct methodologies: immunocapture-based ELISA, NanoSight-based technologies, flow cytometry, and simple protein counts in the EVs purification. It is noteworthy that all the studies got to the same evidence: tumor patients have higher circulating EVs levels than healthy individuals. However, if we want to transfer this approach into the clinical practice we need an agreement on the most reliable, and probably cheap, methodology to be used in clinical cancer laboratories worldwide.

Another significant issue is that the assessment of EVs levels in cancer patients may be improved if implemented with highly specific markers. To this purpose, it is mandatory to go ahead with studies looking at new EVs-related molecules, including proteins, lipids and nucleic acids. However, this approach should start from the evaluation of the specific expression of known tumor markers on EVs. This can well include some scattered observations obtained in few patients, such as that of CEA in colon cancer patients (Huber et al., 2005), but also include many other reports covering virtually all the standard tumor markers exploited in the clinical management of cancer patients (reviewed in Zocco et al., 2014).

Some potential biomarkers are currently under investigation, including survivin, which has been identified as a promising surrogate biomarker for early diagnosis of prostate cancer (Khan et al., 2012). Elevated levels of EV-expressing TYRP-2, VLA-4, HSP70 and HSP90 have been also detected in the plasma of melanoma patients (Peinado et al., 2012). A group of chaperonins, including HSP70 and HSP90, that belong to the family of heat shock proteins (HSPs), has been recently investigated in order to understand their potential role in cancer pathogenesis

and progression. Remarkably, EV-associated levels of HSP60 were dramatically decreased in colon cancer patients after surgically removing the tumor (Campanella et al., 2015). This suggests that one of the most interesting exploitation of circulating EVs quantification is the follow-up of surgical treatment of cancers, both at the diagnosis and following relapses. Remarkable results were obtained by comparing N-glycan profiles of EVs from indolent and aggressive prostate cancer to non-cancer conditions (Nyalwidhe et al., 2013). A series of clinical data of paramount importance for biomarker discovery in body fluids is summarized in Table 1.

In addition to plasma, urine is another body fluid that can be easily exploited in the clinical management of cancer patients, particularly in patients with genitourinary tract malignancies. Interestingly, proteomic analysis of urinary EVs mirrors reliably certain kidney pathologies as demonstrated by Pocsfalvi (Pocsfalvi et al., 2015). The authors showed that polycystin-1 and polycystin-2 expression, together with that of other Ca^{2+} -binding proteins (annexin A1, annexin A2, protein S100-A9, protein S100-A8, and retinoic acid induced protein 3) are significantly altered.

Altogether the available data suggest that quantification and characterization of EVs in human body fluid may be highly helpful as a new non-invasive diagnostic tool for the clinical management of cancer patients. We suggest that quantification EVs circulating mass may represent a very important endpoint to be achieved.

4. Conclusions

Currently available tumor biomarkers are an inefficient and unreliable tool for both tumor screening and the clinical follow-up of tumor patients. While the achievement of valuable non-invasive approach for both diagnosis and prognosis of cancer is a critical and strategic endpoint in the fight against cancer, the results obtained to date are delusive actually. In the last decades a huge amount of data supporting the importance of extracellularly released vesicles in both physiology and pathology has been produced. In particular, a deal of studies has shown that EVs are a natural shuttle for proteins, lipids and nucleic acid (including mRNA and miRNA). This certainly represents an impressive amount of precious information that increases our knowledge on life science, but also provides a novel idea for improving early diagnosis of diseases, specifically of cancer. Cancer and deaths for cancer incidence were remarkably increasing in the last decades (up to 14.1 million new cancer cases and 8.2 million deaths occurred in 2012 worldwide) (Torre et al., 2015). In the absence of a sufficiently effective therapy other than surgery after an early diagnosis (so called “Secondary Prevention”), it is particularly urgent to obtain an efficient, non-invasive, and possibly affordable, new diagnostic tools for cancer. This review proposes the determination of human body fluids EVs levels as the most reliable prognostic and diagnostic tools for both screening and early diagnosis of cancer and cancer recurrence. The real challenge of this perspective review is to address the quantification of the whole circulating EVs

mass as a way to distinguish between patients with cancer and healthy individuals. We here emphasize that presently the EVs related markers did not show cancer type specificity, but they help in quantifying circulating EVs. In our opinion, the measurement of exosome levels in body fluids of patients can be an easy and cheap way to reach the same goal, i.e. early cancer diagnosis and/or follow-up.

The importance of this approach has been recently emphasized in an article commenting the industrial investment in the use of exosomes in cancer diagnostic (Sheridan, 2016). A pool of European scientists in the field, that networked within a COST European Project (European Network on Microvesicles and Exosomes in Health and Disease: MEHAD), has recently published a perspective article proposing nanosized extracellular vesicles as the future of NanoMedicine (Fais et al., 2016), with the aim to emphasize the level of importance of EVs in the future of Medicine as both biomarkers of disease and shuttle for therapeutic molecules.

We know that exosomes may fuse with target cell plasma membranes delivering their contents within the recipient cells (Parolini et al., 2009), and conceivably changing their biology (Cantaluppi et al., 2012). Thus, EVs may represent not only the future biomarkers in medicine (Properzi et al., 2013), but also a very valuable and effective “nanovector” for either chemical or biological drugs, as well (Fais et al., 2013). The horizon of the EVs involvement in our body function is far to be defined. Recently, EVs have been shown to transfer genes to the germ line, thus probably contributing to our genome changes (Cossetti et al., 2014), suggesting that they can well be the ideal vehicle for gene therapy as well. In fact, the possibility of modifying their content by bioengineering methods is another important endpoint of clinical scientists involved in “teranostics”, aiming at using molecules which are the same time tracers and therapeutic agents (Corbin and Zheng, 2007; Dai et al., 2008; Lammers, et al., 2011; Kooijmans et al., 2012; El Andaloussi et al., 2013b; Cooper et al., 2014; Fang et al., 2014). A scheme for the clinical use of exosomes in the diagnostic field is shown in the Graphical Abstract, but we are more than convinced that the clinical use of exosomes is probably without border (Escudier et al., 2005), or at least this is our hope.

In conclusion, the field of *exosomology* is in great expansion and the future appears very promising. Scientific discussion among experts in the field are crucial to reach the goals in a near future, concentrating in optimizing economic resources for the interest of patients. We propose the use of EVs quantification in the body fluids of cancer patients as the definition of “Liquid Tumor Mass” for the early diagnosis of cancer and cancer relapses, as well, and we hope with this perspective article to trigger a fruitful discussion on this issue.

With this TEM pictures we want to summarize the mechanisms of exosomes formation and secretion (Stoorvogel et al., 2002): 1) The inward budding of clathrin-coated micro domains on the plasmatic membrane; 2) The intra-lumen vesicles (ILV) formation, which bud inwards and pinch off into the lumen of the multivesicular bodies (MVBs); 3) The extracellular secretion of the exosomes.

Table 1
Summary of important data showing the utility of exosomes in clinical studies.

Cancer type	Marker(s)	Body fluid	Method	Ref.
Hematological tumors	CD9, CD13, CD19, CD30, CD38, CD63	Serum	FACS	Caivano et al., 2015
Prostate cancer	CD9, CD63	Urine	TR-FIA	Duijvesz et al., 2015
Prostate cancer	Survivin	Plasma	ELISA	Khan et al., 2012
Prostate cancer	–	Plasma	NTA	Nawaz et al., 2014
Prostate cancer	N-linked glycans	Prostatic secretions	MALDI-TOF, HPLC, MS	Nyalwidhe et al., 2013
Colon cancer	Hsp60	Plasma	Western blot	Campanella et al., 2015
Colon-rectal cancer	–	Plasma	FACS	Silva et al., 2012
Melanoma	CD63, caveolin-1	Plasma	ELISA	Logozzi et al., 2009
Melanoma	MIA, S100B	Serum	Electro-chemiluminescence	Alegre et al., 2016
Melanoma	TYRP-2, VLA-4, HSP70, HSP90	Plasma	NTA	Peinado et al., 2012
Pancreatic cancer	Glypican 1	Serum	NTA	Melo et al., 2015
Brest cancer	miR34a, dicer	Plasma	RT-PCR, western blot	Lowry et al., 2015
Gastrointestinal stromal tumor	–	Plasma	FACS	Ogorevc et al., 2013

The upper panel (A) shows an embedded preparation of a cell containing multi-vesicular bodies (MVBs) filled of vesicles of different size, including nanovesicles, in proximity to the plasma membrane (bar 500 nm). Panel (B) shows epon-embedded and sectioned exosomes after secretion with the typical ultrastructural features: the lipid bilayer and range in size from 30 to 100 nm. The picture includes both single vesicles and clusters of vesicles (bar 200 nm). Panel (C) shows some detail of vesicles of different size, ranging from 187 and 80 nm of diameter (bar 200 nm).

Further methodological details: extracellular vesicles were obtained by ultracentrifugation. Briefly, 50 ml of cell-free medium was collected and centrifuged at $13,000 \times g$ for 20 min at 4°C to bring down and eliminate small cellular debris and mitochondrial contaminants. The supernatant was collected, and exosomes were obtained by ultracentrifugation at $110,000 \times g$ for 2 h at 4°C . The pellet was collected and washed once in PBS, resuspended in 100 μl of PBS containing protease inhibitors, and stored at -80°C until use (Campanella et al., 2012). The exosomes suspension was fixed with Karnovsky's fixative and then embedded for electron microscopy.

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