Extracellular vesicles as shuttles of tumor biomarkers and anti-tumor drugs

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Extracellular vesicles (EV) include vesicles released by either normal or tumor cells. EV may exceed the nanometric scale (microvesicles), or to be within the nanoscale, also called exosomes. Thus, it appears that only exosomes and larger vesicles may have the size for potential applications in nanomedicine, in either disease diagnosis or therapy. This is of particular interest for research in cancer, also because the vast majority of existing data on EV are coming from pre-clinical and clinical oncology. We know that the microenvironmental features of cancer may favor cell-to-cell paracrine communication through EV, but EV have been purified, characterized, and quantified from plasma of tumor patients as well, thus suggesting that EV may have a role in promoting and maintaining cancer dissemination and progression. These observations are prompting research efforts to evaluate the use of nanovesicles as tumor biomarkers. Moreover, EVs are emerging as natural delivery systems and in particular, exosomes may represent the ideal natural nanoshuttles for new and old anti-tumor drugs. However, much is yet to be understood about the role of EV in oncology and this article aims to discuss the future of EV in cancer on the basis of current knowledge.

Keywords: extracellular vesicles, exosomes, biomarkers, tumors, teranostics

INTRODUCTION

Extracellular vesicles (EVs) are a mixed population of nanovesicles released by a variety of cells in body fluids, such as plasma, serum, or urine, of both healthy individuals and cancer patients (1, 2). EVs are classified based on their size, which ranges from 40 to 10,000 nm, biogenesis and biomarkers. Exosomes (40–100 nm) originate from the endosomal pathway and they are released by the cells following the fusion of multivesicular bodies (MVB) with the plasma membrane. Because of their origin, exosomes are enriched with scaffolding proteins (tetraspanins), proteins for endosomal trafficking (ESCRT-related proteins/Alix), and protein chaperons (heat-shock proteins). Microvesicles and apoptotic bodies (100–1000 nm) are derived by the outward budding of the plasma membrane. This mechanism enriches the membrane vesicle with phospholipids derived from the inner leaflet of the plasma membrane (e.g., phosphatidylserine). The most recently discovered EVs are large oncosomes (4000–10,000 nm), which derive from bulky protrusions of cancer cells and they are enriched with scaffolding proteins (e.g., caveolin-1) and tissue-degrading enzymes (e.g., metalloproteases). EVs have recently emerged as a promising platform for *both* diagnostic and therapeutic approaches in personalized cancer medicine. Due to their contents that include specific proteins, lipids, and nucleic acids, EVs are now considered shuttles of potential biomarkers for early detection and prognosis of either primary tumors or metastatic lesions. Additionally, EVs may carry biomarkers that are usually detected from invasive tissue biopsies, such as gene mutations for targeted cancer therapies (3). These findings suggest a new perspective for the management of cancer, utilizing EVs as a potential source of biomarkers and transitioning the field to the new concept of "liquid biopsy." Mechanistically, EVs may transfer tumor-related molecules into non-tumoral cells to propagate the disease in both paracrine and systemic manner, or they may act as disposal systems for unwanted molecules, including anti-tumor drugs (4). Growing evidences suggest that these mechanisms may be exploited to develop new cancer vaccines and bio-inspired drug delivery systems (5, 6).

This article critically reviews recent reports on the clinical utility and current limitations of exosomes and microvesicles, generically defined as EVs, as nanoshuttles of biomarkers, anti-tumor drugs, and vaccines, opening new avenues for the clinical management of cancer.

EVs AS SHUTTLES OF TUMOR BIOMARKERS
SCREENING AND EARLY DIAGNOSIS

Biomarkers for cancer screening and diagnosis often display low sensitivity and/or specificity, missing patients with early stage disease (false negatives) or detecting those with no disease (false positives). EVs may offer several potential benefits over current clinical biomarkers. EVs may shuttle both clinically validated biomarkers [e.g., prostate-specific antigen (PSA)] and they are a novel source of proteins and nucleic acids that could be exploited as surrogate biomarkers (7); EVs protect their cargo from the attack of

nucleases and proteases, increasing biomarker half-life, and potentially facilitating sample integrity and downstream molecular analyses (8); EVs are well suited for multiplexed biomarker analyses that may increase sensitivity and/or specificity of the diagnostic assay (8, 9).

Clinical studies for EV-associated cancer biomarkers have been already described and they are summarized in **Table 1**. Logozzi and colleagues performed a retrospective study on EV-associated biomarkers in stage III and IV melanoma patients and they showed increased levels of caveolin-1- and CD63-positive EVs in plasma (2). EV-associated caveolin-1 displayed a sensitivity of 69% and specificity of 96.3% while levels of serum LDH were altered only in 12.5% of patients (2). Mechanistically, EVs may have a prominent role in the pathogenesis of melanoma. Melanoma cells have been shown to release exosome-associated oncoprotein MET to educate bone marrow progenitor cells and promote metastases *in vitro* and *in vivo* (10), and elevated levels of MET and phospho-MET have been detected in melanoma patients (10). Additionally, the authors

showed aberrant levels of EV-associated biomarkers TYRP-2, VLA-4, HSP70, and HSP90 in the plasma of melanoma patients (10). Indeed, HSPs are emerging as another potential source of EV-based cancer biomarkers (11). HSP70 is actively secreted by different types of tumor cells through non-classical protein secretory routes, including EVs, and HSP70-positive EVs have been shown to activate macrophages (12) and natural killer cells (13–15) that act against cancer cells; while, the chaperone HSP90 has been shown to enhance cancer cell migration when is released by EV-derived cancer cells (16).

EVs may be exploited as biomarker shuttles for the early diagnosis of prostate cancer (PCa). Serum PSA and prostate-specific membrane antigen (PSMA) have been found on plasma and urine-derived exosomes, though not validated in a large clinical study (7, 17). In another report, exosomal survivin was identified as promising surrogate biomarker for early diagnosis of PCa (19). Plasma levels of survivin-positive-EVs were higher in PCa patients than benign hyperplastic patients and healthy donors, potentially

Table 1 | Pre-clinical and clinical studies on EV-shuttled biomarkers.

Cancer biomarker	Disease	Indication	Biofluid	Clinical study size	Reference
PSA	Prostate cancer	Screening/early diagnosis	Urine	Controls $N = 10$; disease $N = 24$	(7)
PSA	Prostate cancer	Screening/early diagnosis	Plasma	Control $N = 2$; disease $N = 5$	(17)
EGFRvIII	Glioblastoma	Early diagnosis	Serum	disease $N = 30$	(18)
(phospho)Met	Melanoma	Early diagnosis/prognosis	Plasma	Controls $N = 7$; stage III $N = 24$; stage IV $N = 14$	(10)
Caveolin-1	Melanoma	Early diagnosis	Plasma	Controls $N = 58$; disease $N = 90$	(2)
Survivin	Prostate cancer	Early diagnosis	Plasma	HD $N = 8$; BPH $N = 20$; disease $N = 39$	(19)
CD 24	Breast cancer	Early diagnosis	Serum	HD $N = 14$, disease $N = 18$	(20)
EGRF	Lung cancer	Diagnosis/personalized medicine	Serum	HD $N = 9$; disease $N = 9$	(21)
miR-21, miR-141, miR-200a, miR-200b, miR-200c, miR-203, miR-205, miR-214	Ovarian cancer	Early diagnosis/prognosis	Serum	HD $N = 10$; stage I $N = 10$; stage II $N = 10$; stage III $N = 20$; stage IV $N = 10$	(8)
RNU6-1, miR-320, and miR-574-3p	Globlastoma	Early diagnosis	Serum	Controls $N = 50$; disease $N = 50$	(22)
TMPRSS2:ERG2 and PCA3 mRNAs	Prostate cancer	Early diagnosis	Urine	Blinded prospective study $N = 30$	(23)
let-7a, miR-1229, miR-1246, miR-150, miR-21, miR-223, and miR-23a	Colorectal cancer	Early diagnosis	Serum	Controls $N = 22$; disease $N = 88$	(23)
miR-151a-5p, miR-30a-3p, miR-200b-5p, miR-629, miR-100, and miR-154-3p	Lung cancer	Early diagnosis	Plasma	HD $N = 10$; benign disease $N = 10$; malignant disease $N = 10$	(24)
TGFB1 and MAGE3/6	Ovarian cancer	Prognosis/therapy monitoring	Plasma	HD $N = 10$; benign disease $N = 10$; malignant disease $N = 22$	(25)
TYRP2, HSP70, HSC70, VLA-4	Melanoma	Prognosis	Plasma	HD $N = 9$; stage I $N = 2$; stage III $N = 7$; stage IV $N = 18$	(10)
miR-21	Human esophageal cell carcinoma	Prognosis	Serum	HD $= 41$; disease $N = 51$	(26)
KRAS	Pancreatic cancer	Personalized medicine	Serum	HD $N = 2$; disease $N = 2$	(27)
BRAFV600E, EGFR	Lung cancer, melanoma	Personalized medicine	Plasma	*In vivo* model $N = 8$	(3)

providing an alternative tool to reduce the number of false positives generated by the PSA test (19). Analysis of the EV cargo may allow the repositioning of a clinically validated biomarker to a new diagnostic indication. For example, the FDA has recently approved a test (commercialized by Hologic) that supports the clinical decision of repeating a biopsy in suspected PCa patients. The test is based on QRT-PCR detection of PCA3 mRNA from urine collected after digital rectal examination (DRE) and it is not recommended for the early diagnosis of PCa. Instead, a report from Dijikstra and colleagues suggested that the ratio between the levels of EV-associated PCA3 and PSA mRNAs might be useful for the early detection of PCa (9).

EVs have also been evaluated as diagnostic platforms for multiplexed approaches. Seminal work by Taylor and colleagues identified the first disease-specific miRNA signature in EVs derived from ovarian cancer patients (8). They identified a tumor-specific signature of eight miRNAs in EpCam-positive-EVs that discriminated ovarian cancer from benign ovarian disease (8). Remarkably, miRNA levels were not altered by pre-analytical variables such as collection and storage time (8). In another retrospective study, a diagnostic signature of miRNAs was found and validated in serum derived EVs from colorectal cancer patients (23). Sensitivity of the signature was higher than 90%, while serum biomarkers CEA and CA19-9 displayed sensitivities of 30.7 and 16%, respectively (23). These studies provide some level of comfort to support further research around the clinical use of EVs as biomarkers for screening and early diagnosis of cancer. However, they suffer from the lack of standardized protocols of sample collection and storage and the limited sample size (see **Table 1**). For the former, some general consensus was recently achieved by the International Society for Extracellular Vesicles (ISEV), though not fully implemented in clinical studies yet (28). For the latter, large regulated multi-center clinical studies are needed to validate the use of EVs for potential diagnostic applications.

CANCER PROGNOSIS AND PERSONALIZED MEDICINE

EV-based diagnostics may be a practical alternative to tumor biopsy diagnostics that are currently used for prognostic and "personalized medicine" indications. Indeed, EVs can be collected with minimally invasive procedures from a variety of body fluids; they may be more representative of the intra-tumor heterogeneity than fine needle biopsy, thus potentially revealing aggressive primary tumor features and distant metastases (25); EVs may allow the real-time monitoring of therapeutic responses and development of resistance mechanisms to targeted therapies where the mutation status is needed for patient stratification; EVs cargo may complement the use of other emerging "liquid biopsy" platforms such as circulating tumor DNA or circulating tumor cells (27). **Table 1** summarizes the clinical studies on EV-based biomarker for prognosis, monitoring and personalized medicine. These studies lack the sample size and standard operating procedures required for clinical validation. However, the established presence of the disease may facilitate discovery and validation of cancer-derived biomarkers in these indications. From the regulatory perspective, we expect the initial development of EV-based laboratory developed (LDT) tests, which must meet the regulatory

standard of the clinical laboratory improvement act (CLIA) and do not require to go through the FDA approval process. Though the FDA has not approved EV-based tests yet, both industrial and regulatory entities have expressed their interest in developing EV-based tests for personalized cancer medicine. There are several compelling reasons for it. EVs may represent a minimally invasive platform for the development of companion diagnostic (cDx) tests for targeted therapies. Cancer-derived EVs may be exploited for the development of blood-based cDx for cetuximab (Erbitux) since they carry both the drug target (epidermal growth factor receptor) and the mutated *KRAS* gene, which correlates with poor therapeutic responses (21, 27). Cancer-derived EVs may also shuttle genomic DNA with the mutation BRAFV600E, which may be used to develop a cDx test to identify melanoma patients eligible for the treatment with Vemurafenib (3). Moreover, personalized medicine has become one of the fastest growing segments in the molecular diagnostic market due to FDA's recent recommendation of developing cDx tests for approval of new drugs. From the perspective of diagnostic developers, cDx tests are very attractive since they may benefit from fast-track approval and positive clinical adoption. Finally, drug developers may decide to directly reimburse the cost of cDx test to drive clinical adoption and sales of the targeted therapy, thus relieving insurances and patients from a significant economic burden.

TECHNOLOGICAL CHALLENGES IN THE DEVELOPMENT OF EV-BASED DIAGNOSTICS

The field of EVs has evolved over the past 5 years especially in the technical ability to measure genomic and proteomic EV-associated biomarkers. ELISA-based technologies have been the gold standard assay for single-plexed detection of low abundant proteins (down to nanograms per milliliter levels) with high sample throughput. Conversely, multiplexed technology platforms based on mass spectrometry (MS) have been employed for proteomic analyses of cancer-derived EVs but offered rather low sample throughput and detection limits (29). Additionally, the sheer complexity of a biomolecule samples, such as blood plasma specimen further complicates any proteomic analysis. Typical blood samples can contain more than 10,000 different protein species, with concentrations varying over nine orders of magnitude. Such diversities of proteins, as well as their huge concentration ranges, present a formidable challenge for sample preparation in proteomics. Conventional protein analysis techniques, based on multidimensional separation steps and MS, fall short because of the limited separation peak capacity (up to 3000) and dynamic range of detection ($\sim10^4$). Techniques for gene expression analysis have also their limitations. Microarrays afford high gene density and potentially high throughput but they are limited in sensitivity and dynamic range compared to RT-PCR, thus preventing discovery and analysis of many genes potentially adding power to a cancer signature.

Currently, there are four technological challenges when working with EVs: (i) the lack of standardized methods for the isolation of tumor-derived EVs. Current EV isolation protocols are largely based on non-specific physical and chemical properties, such as,

size, density, and solubility as summarized in **Table 2**. These methods are inefficient, time consuming and costly, and produce EVs of variable in yield, purity (origin), and integrity, all features that make them poorly compatible with routine use for diagnostic purposes; (ii) the need for instrumentation capable of detecting EV biomarker panels for improved accuracy of patient diagnosis; (iii) the need for surface chemistry which enables EV biomarker detection from undiluted blood samples thereby providing maximal sensitivity; and (iv) the need for low-cost sensor platforms with high sensitivity and specificity.

EVs AS SHUTTLES OF ANTI-TUMOR DRUGS AND CANCER VACCINES

Nanobiomedicine seeks to exploit the improved (and often novel) physical, chemical, and biological properties of materials at the nanometric scale with a biomimetic approach. EVs may be exploited as biomimetic nanoshuttles of drugs and cancer vaccines for several reasons (5, 33). EVs have a key role in many mechanisms promoting cancer development and progression, including cell–cell communication (34), cell migration (16), metastasis (33), angiogenesis (18), and drug resistance (5). EVs can either bind to membrane receptors or directly interact with internal compartments of the targeted cell to alter cellular behavior and their functions (35–38). Selected subpopulations of EVs or exosome-inspired biomimetic vesicles may be used to deliver anti-tumor drugs to the tumor site (4, 5). Pre-clinical and clinical studies showed that inhibition of tumor acidity induces chemosensitization in cancer patients (4, 39, 40). Since cancer exosomes act as disposal system for chemotherapic drugs, pharmacological inhibition of microenvironmental acidity may increase both exosomes targeting to the tumor site and exosomes uptake by tumor cells, by simply different electrostatic cargos (4, 39, 40). EVs may mediate gene delivery without inducing adverse immune reactions since they are amenable to autologous delivery across tissue barriers, including the blood brain barrier (41–43). Furthermore, EVs may be engineered to carry a variety of biomolecules including therapeutic RNAs (44–53), small molecules (4, 52, 53), and protein or peptide ligands (54). Finally, immune cell-derived EVs may be exploited to develop cancer vaccines. Human natural killer cell-derived exosomes have shown anti-tumor properties *in vitro* and *in vivo* (54, 55). Dendritic-derived exosomes (Dex) carry peptide-MHC complexes that can be transferred to recipient cells and express tumor-derived peptides that induce potent cytotoxic T lymphocytes (CTL)-mediated responses, leading to the regression of established tumors in mice (6, 56). Good manufacturing laboratory (GMP) procedures for exosome harvesting and purification have been set up for clinical implementation

(57) and EVs have been approved for use in clinical trials with the first phase I clinical trial that started at the end of 2000, 30 months after Zitvogel's group publication (6). In the first clinical study, patients underwent leukapheresis and exosomes were purified from monocyte derived-DC (MD-DC) in a GMP setting (58). Dex were loaded with MAGE A3 peptides, administered to 15 stage III/IV melanoma patients through four intradermal vaccinations at 1 week intervals to promote anti-tumor immunity. This study demonstrated, for the first time, the feasibility and safety of Dex-based vaccination in melanoma patients (58). In a similar clinical trial, autologous exosomes were injected weekly into 13 non-small-cell-carcinoma lung cancer (NSCLC) patients for 4 weeks but they induced weak immune responses against the tumor (59). These low immunogenic capacities have led to the development of second-generation Dex with enhanced immune-stimulatory properties (60). Viaud et al. reported the clinical grade manufacture of large-scale interferon-γ-Dex vaccines currently used in a phase II trial testing the clinical benefit of Dex as a maintenance immunotherapy in inoperable (stage IIIB to IV) NSCLC patients that responded to chemotherapy. Dex was purified from autologous maturing MD-DC loaded with MAGE-3 and NY-ESO-1, MAGE-1, MAGE-3, MART-1 restricted peptides (60). Patients received four cycles of platinum-based chemotherapy followed by a combination of a 3-week oral therapy with low dose cyclophosphamide followed by four weekly intradermal Dex injections. The study was launched in November 2009 at the Gustave Roussy and Curie Institutes and the results are not yet published (60). Another clinical study used ascites-derived exosomes (Aex) as immunomodulatory agents. In a phase I clinical trial, Aex were evaluated alone or in combination with the granulocyte-macrophage colony-stimulating factor (GM-CSF) for the immunotherapy of colorectal cancer (61). The Aex isolated by sucrose/D(2)O density gradient ultracentrifugation contained the diverse immunomodulatory markers of exosomes and tumor-associated carcinoembryonic antigen (CEA). Both therapies were safe and well tolerated with mild adverse events (grade 1–2) causally related to the use of Aex or Aex plus GM-CSF. Importantly, Aex plus GM-CSF but not Aex alone induced beneficial tumor-specific anti-tumor CTL responses (61).

Despite these promising results, the use of EVs as shuttles of anti-tumor drugs and anti-cancer vaccines is far from clinical acceptance. The lack of standardized protocols for isolation of clinical grade EVs (sub)populations and the partial understanding of the mechanisms involving EVs in cancer have so far hampered the number and the size of these clinical studies.

Table 2 | Methodologies for the isolation of EVs.

EV isolation method	Category	EV type	Reference
Ultracentrifugation	Physico-chemical	Total exosome population	(18)
Filtration (0.22 μm) and ultracentrifugation	Physico-chemical	Total exosome population	(30)
Sucrose gradient	Physico-chemical	Total exosome population	(31)
ExoQuick precipitation	Physico-chemical	Total EVs population	(32)
Immunocapture with magnetic beads with anti-EpCam antibodies	Immuno-based	EpCam-positive-EVs	(8)

CONCLUSION

Extracellular vesicles have the potential to revolutionize the clinical management of cancer. EVs carry a plethora of validated and surrogate biomarkers with diagnostic, prognostic, or cDx value. Among the surrogate biomarkers, molecular chaperones are likely to yield useful diagnostic and prognostic biomarkers as well as anti-cancer molecules. Despite the accumulating evidences, most of the clinical studies lack the statistical power required for biomarker validation and clinical adoption. Furthermore, the biogenesis of EVs during tumorigenesis is largely unknown, especially in the early phases of the disease. For this reason, prognosis and therapy follow up may offer a relatively easier route to biomarker validation than early diagnosis given the established presence of the disease (or absence in the case of post-surgery/treatment groups). EVs are also an intriguing source of biomarkers for personalized medicine. However, they will need to be evaluated against other "liquid biopsy platforms" such as circulating tumor DNA and circulating tumor cells, which already have established protocols for isolation from body fluids (62, 63). Detection of EV-associated biomarkers from plasma or other complex biofluids is still quite challenging since sensitive, multiplexed assays are not cost effective, robust, and high throughput enough to drive clinical adoption. Finally, several preliminary studies indicate that autologous EVs as promising delivery systems for drugs and cancer vaccines due to their ability to target the disease with only minimal side effects. More studies are warranted to test the utility of EVs as natural carriers of biomolecules for therapeutic purposes.

5-YEAR PERSPECTIVES

This new area of nanobiomedicine focuses on multi-disciplinary research to build new systems for various nanobiomedical applications, ranging from the medical use of nanoplatform-based diagnostic agents, to therapeutic agents and even possible future applications of diagnosis and therapy – theranostics. One of the most important theranostic strategies is the nanoformulation of new agents based on an "all in one approach." In this perspective, EVs can be considered potential nanocarriers of both diagnostic/prognostic biomarkers and therapeutic molecules. In the next 5 years, we expect that larger multi-center studies will be performed for the clinical validation of EV as autologous nanovectors for diagnosis, prognosis, and cancer therapy. Hopefully, these efforts will shed some light on the real value of the clinical use of EVs. We also expect EVs to have an impact in personalized cancer medicine with the introduction of cheap, multiplexed, and robust analytical assays and the growing interest of large pharmaceutical companies, regulatory agencies, and governments to reduce patient's therapeutic burden and healthcare costs.

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