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Extracellular vesicles as a platform for 'liquid biopsy' in glioblastoma patients

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Abstract

Extracellular vesicles (EVs) are cell-secreted vesicles that range from 30–2000 nm in size. These vesicles are secreted by both normal and neoplastic cells. Physiologically, EVs serve multiple critical biologic functions, including cellular remodeling, intracellular communication, modulation of the tumor microenvironment and regulation of immune function. Because EVs contain genetic and proteomic contents that reflect the cell of origin, it is possible to detect tumor-specific material in EVs secreted by cancer cells. Importantly, EVs secreted by cancer cells transgress anatomic compartments and can be detected in the blood, cerebrospinal fluid, and other biofluids of cancer patients. In this context, there is a growing interest in analyzing EVs from the biofluid of cancer patients as a means of disease diagnosis and therapeutic monitoring. In this article, we review the development of EVs as a diagnostic platform for the most common form of brain cancer, glioblastoma, discuss potential clinical translational opportunities and identify the central challenges associated with future clinical applications.

Keywords

biomarker; exosomes; extracellular vesicle; glioma; microvesicle

Glioblastoma is the most common form of primary brain neoplasm. Despite aggressive surgical resection, chemotherapy and radiation therapy, the median survival for glioblastoma patients is approximately 14 months [1,2]. Optimal management of glioblastoma patients is limited by a lack of effective strategies for monitoring response to therapy [3]. In the current clinical practice, therapeutic responses are monitored through serial neurologic examinations and MRI studies. However, both forms of assessment are crude measures of the underlying disease status.

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Monitoring the clinical status of the patient will detect disease progression only after sufficient tumor burden has accumulated to alter the neurologic examination. This level of disease burden occurs long after the tumor cells have acquired resistance to therapy. While MRI potentially shortens this delay, the resolution limit of MRI renders this modality insensitive. Current MRI has a resolution limit of approximately 2–3 mm [4]. Considering that the size of a tumor cell is on the order of 10 μm, tumor cells will undergo more than 20 rounds of exponential growth before detection is feasible by MRI. Moreover, modalities used to treat glioblastoma (radiation and chemotherapy) often induce changes in the brain that mimic those of tumor progression on MRI [5]. While advanced MR modalities have been developed to better detect tumor progression (reviewed in [6,7]), the time required for the scheduling, acquisition and interpretation of these studies further delay timely intervention. Though brain biopsy and histologic analysis can definitively evaluate disease progression, serial brain biopsy is both invasive and impractical given cumulative surgical risk [8].

The delay in the detection of therapeutic failure is particularly problematic since the standard chemotherapy used to treat glioblastoma, temozolomide, is a mutagenic agent [9]. Temozolomide induces tumor kill by alkylating the O6 position of the guanine nucleoside, resulting in stalled replication fork that ultimately triggers cell death [10,11]. However, in cells that have acquired resistance to temozolomide, the O6 methylated guanine can mispair with the thymine nucleoside, resulting in G:C → A:T transitions [12]. This mutagenesis is expected to promote the emergence of more aggressive clones [13], thereby limiting the efficacy of subsequent therapeutic regimens [14]. Thus, temozolomide treatment should be terminated as soon as resistance against this agent can be detected.

In these contexts, there is a critical need in the development of diagnostic tools that would afford timely assessment of disease burden and therapeutic response in glioblastoma patients. There is a growing interest in extracellular vesicles (EVs) as a biomarker platform in this development. Recent studies have demonstrated that glioblastoma cells secrete EVs that harbor tumor-specific mRNAs, miRNAs, and proteins (Figure 1) [15–18]. These EVs transgress multiple anatomic compartments and can be detected in the blood [18] and CSF [15] of glioblastoma patients. Pilot studies suggest that the analysis of EVs derived from the biofluid of glioblastoma patients may serve as a ‘liquid biopsy’ platform [19].

Extracellular vesicles: overview of their biology, nomenclature & isolation

Biology

EVs play a key role in several biological processes. For the secreting cells, EVs facilitate cellular membrane remodeling, recycling and the removal of cellular components [20–24]. For instance, during reticulocyte maturation, the cell transforms from a spherical volume into a biconcave structure, a process that requires the removal of large amount of membrane and proteins from the cell surface. This removal is mediated through shedding of cellular membranes and components through EV secretion [23]. Similarly, EV secretion is the major mechanism by which eggs avoid polyspermy during fertilization. Mammalian fertilization is mediated through interaction between Izumo1, a sperm protein, and Juno, an egg surface

receptor. Rapid shedding of Juno through EV secretion occurs rapidly after fertilization as to ensure that each egg fuses with a single sperm [21].

For the cells in the immediate vicinity of the EV-secreting cells, EVs mediate intercellular communication and transport membrane bound receptors, nucleic acids and other proteins to target cells [25–27]. For instance, oligodendrocytes and astrocytes secrete EVs that deliver specific proteins, mRNA and miRNA to surrounding neurons to provide trophic support. Similarly, neurons secrete EVs that are taken up by astrocytes and oligodendrocytes [25], thereby reciprocally influencing the functions of these cells.

In terms of microenvironment modulation, secreted EVs facilitate remodeling of the extracellular matrix [22,28–31]. Tumor microvesicles showed an enrichment of matrix degrading metalloproteases (MMP2, MMP9, MTI-MMP and urokinase-type plasminogen activator) [28–31]. These secreted EVs play critical roles in modulating the migration and invasion of cancer cells [32,33]. Moreover, EVs released by metastatic cells interact with bone marrow-derived cells to promote a pro-vasculogenic and pro-metastatic phenotype and to facilitate the establishment of the pre-metastatic niche [34].

Finally, EVs can play an important role in immunomodulation [22]. Depending on the cellular context, EVs can induce both immunostimulatory and immuno-suppressive effects [35–37]. Dendritic cells release EVs that augment immunologic responses by activating T cells [35] or transferring MHC molecules to other dendritic cells [36]. On the other hand, EVs isolated from tumor cells have been shown to induce the expansion of human T-regulatory cells and suppress the immune responses [37].

Nomenclature

EVs have been categorized into exosomes, microvesicles, apoptotic bodies, retroviral particles and other entities based on the mechanism of biogenesis, and an in-depth review of this topic can be found elsewhere [20]. In brief, exosomes refer to vesicles of 30–100 nm in size and arise from the endosomal network, a membranous compartment responsible for sorting various forms of intraluminal vesicles. Microvesicles refer to larger sized vesicles (50–2000 nm) that arise as a result of direct budding from the plasma membrane. Retrovirus-like particles are 90–100 nm vesicles that also arise from direct budding of the plasma membrane. In contrast to microvesicles, however, the mechanism of formation is driven by retroviral proteins, such as Gag [38]. Finally, apoptotic bodies refer to membrane-enclosed vesicles that are formed during apoptosis. These vesicles range between 50 and 4000 nm in size.

As above discussed, the terms ‘exosomes’, ‘microvesicles’, ‘apoptotic bodies’ and ‘retroviral particles’ each hold a unique significance based on their respective mechanisms of biogenesis. Unfortunately, surface markers that uniquely distinguish one EV sub-population from another have not yet been established. As such, it is the currently not possible to discriminate the mechanism of biogenesis when analyzing EVs derived from clinical specimens.

In this context, alternate nomenclatures have been loosely applied when discussing EVs isolated from clinical specimens. One nomenclature convention adopted was to name the vesicles based on the source of isolation. Terms such as oncosomes, prostatsomes and epididimosomes were coined [20]. EVs have also been classified based on size. In general, the term exosome refers to EVs <100 nm in size, whereas the term microvesicles refers to exosomes 100 nm–2 μm in size [20]. It is important to note that though there is some consensus in the literature regarding the size-based definitions, precise cut-offs are arbitrary and vary depending on the author. For the purposes of this review, we will refer to vesicles isolated from clinical specimens as EVs, while reserving the terms exosome and microvesicle for vesicles derived from model systems where biogenic mechanisms can be clearly defined.

Isolation & characterization

EVs have been isolated from blood, urine, CSF, lymphatics, tears, saliva and nasal secretions, ascites and semen. As indicated above, multiple methods have been used to isolate EVs. Some groups use differential centrifugation or density gradients to remove larger cellular debris and isolate EVs [15,39,40]. Other reported methods include chemical precipitation, the use of serial filters [41], EV-binding resins, immuno-isolation with magnetic beads [42,43], and microfluidic separations [17]. It is important to note that the biologic properties and contents of the EVs are greatly influenced by the method of isolation [44,45]. In this context, it is important to specify the method by which EVs are isolated in scientific communications.

Due to their small size, the analysis of EVs presents significant technical challenges. Large EVs (>300 nm) can be analyzed by flow cytometry [46]. Newer generation of cytometry instruments, including the Apogee (Apogee Flow Systems), Gallios (Beckman Coulter) and BD-Influx (Becton Dickinson) may be used to sort EVs down to 200 nm in size [47]. Current efforts are directed towards the development of cytometric instruments that can afford sorting of smaller particles.

Currently, EVs smaller than 200 nm are not amenable to flow cytometric analysis. The most frequently used descriptive analytic for these particles involve direct visualization using electron microscopy [15,48]. Nanoparticle tracking analysis is another method for quantifying the distribution of EVs and calculating particle size [49,50]. Nanoparticle tracking analysis uses a digital camera to capture the movement of EVs over a series of frames. The rate of the particle movement is then used to calculate particle size. Resistive pulse sensing is another method for quantifying EV size. Two cells are separated by a membrane containing a single pore. As EVs pass through the pore, a transient change in ionic current flow is detected and used to calculate the volume of the EV [51]. Other analytic techniques based on surface markers such as diagnostic MR, ELISA and western blotting have also been reported [20].

EV as a biomarker platform for glioblastoma

High-throughput genomic technologies have revolutionized our understanding of glioblastoma and identified novel biomarkers, with potential clinical application. In pre-

clinical models, EVs isolated from glioblastoma cell lines contain tumor-specific mRNA and miRNAs [15,16,18]. Importantly, these markers can also be detected in EVs derived from the biofluid of glioblastoma patients. As such, the disappearance of these biomarkers from the patient's biofluid may serve as proxy for therapeutic response. By the same rationale, re-emergence of these biomarkers may indicate disease recurrence. In the ensuing section, four studies describing the applications of this strategy will be reviewed.

EGFRvIII

The EGFRvIII mutation is found in 20–30% of all glioblastomas [52,53]. The EGFRvIII truncation mutant arises from the deletion of wild-type EGFR exons 2 through 7 [53]. EGFRvIII mutants express a constitutively active receptor that plays a significant role in glioblastoma pathogenesis and development [54]. Importantly, the EGFRvIII mutation is not found in normal tissue, thereby rendering EGFRvIII ideal as a tumor biomarker.

In a study by Skog *et al*., the EGFRvIII status of EVs derived from the sera of 30 glioblastoma patients were examined by qRT-PCR. EGFRvIII was found in the EV derived from five patients harboring EGFRvIII-expressing glioblastoma. Interestingly, two patients with biopsy specimen scoring negative for EGFRvIII harbored EGFRvIII in their sera EVs, suggesting the initial biopsy may have targeted a non-representative area in a histologically heterogeneous tumor. However, PCR contamination leading to a false-positive signal cannot be excluded given the study design. Of the patients who scored positive for EGFRvIII in sera EVs, longitudinal sera collection was performed in five of these patients. In all cases, EGFRvIII was undetectable after resection [18].

IDH1R132H

Mutations in isocitrate dehydrogenase 1(IDH1), a citric acid cycle enzyme, are found in approximately 10% of all glioblastomas and 80% of secondary glioblastomas [55]. The native function of the protein catalyzes the oxidative decarboxylation of isocitrate to α-ketoglutarate [56]. The majority of IDH1 mutated tumors exhibit an arginine to histidine mutation (R132H) [55]. The resultant enzyme is deficient in its native function but harbor a new catalytic activity – the production of 2-hydroxyl-glutarate. The production of 2-hydroxylglutarate is associated with an altered chromatin state [57], which is thought to contribute to glioblastoma pathogenesis. However, the molecular mechanisms underlying this pathogenesis remain poorly understood. Despite a lack of clarity in these mechanisms, IDH1 mutation status has come to serve as a means of differentiating secondary glioblastomas (i.e., tumors that arise from lower grade gliomas) from primary glioblastomas that arise *de novo*.

A study by Chen *et al*. explored the hypothesis that mutant transcripts of IDH1 may be detected in EVs isolated from patient CSF [16]. The authors utilized highly sensitive digital PCR platform, with primers designed to specifically detect the mutated IDH1 sequence. Parallel experiments were performed to assess the IDH1 status of glioblastoma specimens with matched CSF collected from the same patient. While the authors were not able to detect mutant IDH1 transcript in the sera of patients afflicted with IDH1 mutated tumors, the detection of mutant IDH1 RNA transcript was achieved in five of eight CSFs derived from

patients with IDH1 mutated tumors. Importantly, the absolute quantity of mutant IDH1 transcripts directly correlated with the tumor volume, suggesting the feasibility of using EV-based platform to assess glioblastoma disease burden.

miR-21

miRNAs are small RNA molecules that help regulate gene expression by silencing mRNA transcripts with partially complementary sequences. Given the inherent stability of miRNAs relative to mRNAs and their presence in EVs, EV miRNAs represent particularly attractive biomarker platform for 'liquid biopsies'. miR-21 is a miRNA that is highly over-expressed in glioblastoma cells [58]. The expression of miR-21 in glioblastoma mediates several essential oncogenic functions, including suppression of apoptosis, growth proliferation and tolerance of DNA damages [59]. Akers *et al*. showed that miR-21 levels in EVs isolated from the CSF of glioblastoma patients were 10-fold higher than those isolated from controls. Importantly, using CSF-derived EV miR-21 levels, the authors were able to prospectively distinguish CSF derived from glioblastoma and non-oncologic patients. Furthermore, miR-21 levels in CSF EVs decreased by an order of magnitude after surgical resection of the glioblastoma in patients with available longitudinal follow-up [15].

Combined proteomic platform

Shao *et al*. developed a micro-fluidic chip technology that used magnetic nanoparticles to label EVs followed by analysis with a micro-nuclear MR system [17]. This technology allowed the detection of EV proteins with a sensitivity exceeding other protein detection methods (including western blotting and ELISA) by several orders of magnitude. The authors used this μNMR technology to measure EGFR, EGFRvIII, podoplanin and IDH1 R132H molecules in EV isolates from the sera of 24 glioblastoma patients and eight healthy volunteers. In this study, patients with higher levels of tumor-related molecules (such as EGFR, EGFRvIII and podoplanin) in their EVs were more likely to fail standard temozolomide/radiation treatment ($p < 0.005$).

Expert commentary

Significant strides have been made in the understanding of EVs and the role they play in cellular physiology and intercellular communications. The critical observation that glioblastoma cells secrete EV-containing genetic materials that mirror the intracellular tumor milieu has laid the foundation for EVs as a platform for 'liquid biopsy'. With the uncovering of the genomic landscape of glioblastomas and the development of highly sensitive detection technologies, such as digital PCR, maturation in the clinical application of this platform is expected. Early reports suggest the EV platform for 'liquid biopsy' is promising. However, there are major challenges that require creative and thoughtful solutions before clinical translation. These issues are outlined below.

Since non-neoplastic cells secrete EVs and these cells outnumber neoplastic cells by several orders of magnitude in a patient, tumor-specific EVs remain a rarity in clinical samples. This rarity limits the sensitivity of EVs as a biomarker platform. While one method for bypassing this limitation is to analyze large volumes of the biofluid, such an approach may not be

practical. As such, there is a critical need to identify reagents that enrich the yield of glioblastoma-specific EVs from smaller samples. To this end, two major advances are needed. First, surface markers that enhance the isolation in glioblastoma-specific EVs will need to be identified. Second, affordable technologies for the reliable and efficient sorting of EVs of all size ranges will need to be developed. If such technologies become widespread, they may also enhance communication and cooperation within the EV field by facilitating the development of standardized inter-institutional protocols.

It is also conceivable that certain genetic materials, such as miRNA, may be preferentially concentrated in glioblastoma EVs. If so, the analysis of these enriched genetic materials may further enhance the inherent sensitivity of the assay. Since the mechanisms responsible for transporting genetic materials from the cellular context into the EVs remain poorly understood, selection of the optimal assay for EV analysis will likely require empiric determination.

Quantitative analysis of the genetic contents of EVs is another issue that requires thoughtful consideration. Quantitative assessment of mRNA or miRNA expression in the cellular context typically involves normalization of the query gene to housekeeping genes, such as glyceraldehyde-3-phosphate dehydrogenase or 18S. These housekeeping genes are expressed at high levels, exhibit little cell-to-cell variation and serve as a proxy for the total number of cells analyzed. Unfortunately, the quantities of various housekeeping genes are rare and highly variable among EVs [15]. As such, random biases would be introduced if the query transcript level is normalized to an arbitrarily selected gene, such as glyceraldehyde-3-phosphate dehydrogenase. In this context, we favor PCR quantitation of the genetic material in absolute terms [15]. However, the question remains: should this absolute quantity be normalized to the total volume of the input biofluid, to the total protein content of the EVs, to the total number of EVs or to highly abundant transcripts in the EVs. Much work remains to be done to address this important question.

Another important issue that is at the core of any biofluid-based ‘liquid biopsy’ involves the dynamic nature of the biofluid. Like one’s blood pressure, samples of biofluid taken at any one moment may not be identical to those taken at another moment. The contents of the biofluid (CSF, blood or otherwise) are subject to changes related to natural circadian rhythm, food intake, medications, exercise and a plethora of other physiologic and iatrogenic factors. To further confound the matter, the method by which the biofluid is collected significantly impact the quality and quantity of EVs collected. Agitation of the collected fluid and the time interval between collection and processing of the samples are also equally important factors [47]. Given the inherent biologic complexity of the various biofluids, careful consideration should be given to the optimal method of collection – which will likely differ depending on the intended assay and biomarker.

While the initial studies of EV as a biomarker platform for glioblastomas show promise, validation through larger sample sets and prospective clinical trials are the necessary next steps. Given the rarity of glioblastomas, such efforts require multi-institutional collaborations and infrastructures. A pilot effort toward this end has been initiated by the Accelerated Brain Cancer Cure Foundation, an effort that involved 18 academic

neurosurgical centers across the U.S. Undoubtedly, the optimal technical platform for EV analysis will continue to evolve during the course of this prospective study. As such, future clinical trials should be designed with an adaptive intent, with the possibility of comparing evolving methods of EV isolation and analysis. The trial design should also afford opportunities for comparing EV-based platforms to other established and emerging biomarker platforms such as circulating tumor cells [60] or cell-free DNA [61].

In conclusion, the concept of EV-based 'liquid biopsy' for monitoring disease status is promising. The preliminary reports exploring this concept with application to glioblastoma patients are compelling. Successful implementation of this platform can fundamentally alter the paradigm of care for glioblastoma patients and make meaningful gains in terms of improvement in clinical outcomes. Ultimately, thoughtful clinical trial design and technology advancements will be needed to harness the potential of EVs for this clinical translation.

Five-year view

Over the course of the next 5 years, priorities in EV research, as it pertains to biomarker development, should be placed on optimizing methods of isolation with the recognition that chemical and biologic content of clinical biofluids necessarily differ from those of cell culture supernatants. Optimal methods of isolation likely will require glioblastoma-specific EV markers as a function of the source of the biofluid. Given the spectrum of genetic materials present in biofluid EVs, the analytic of maximal utility remains a central question. Moreover, normalization and quantitation of genetic materials isolated from biofluid EV is an unresolved question. The solution to these challenging questions will likely involve empiric determination rather than theoretical considerations. Spontaneous fluctuation in the contents of patient biofluid as well as perturbation imposed by iatrogenic or iatrogenic poses another major challenge as they inevitably introduce 'noise' to the biomarker analytic. As such, efforts to mitigate these dynamic 'noises' will be needed, including defining strict collection protocol. With maturation of EV biomarker analytics in the upcoming years, there will be increasing need for clinical validation and comparison to other biomarker platforms, including circulating tumor cells. Thoughtful consideration in clinical trial design and multi-institutional collaboration are warranted in this regard. The challenges facing EV biomarker development are daunting but not insurmountable. Collegial and deliberate collaborative endeavors are undoubtedly required to address these central challenges.

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Key issues

- Diagnosis and therapeutic monitoring remains a major challenge for neuro-oncologic diseases, such as glioblastomas.
- Extracellular vesicles (EVs) are cell-secreted vesicles that are 30–2000 nm in size. EVs contain genetic and proteomic contents that reflect the cell of origin.
- Glioblastoma cells secrete EVs that harbor tumor-specific mRNAs, miRNAs and proteins. These EVs transgress multiple anatomic compartments and can be detected in the blood and cerebrospinal fluid of glioblastoma patients.
- Pilot studies have demonstrated that glioblastoma disease burden and therapeutic responses associate with protein, miRNA and mRNA profiles of EVs isolated from the biofluids of glioblastoma patients.
- Optimization and validation of results provided by the pilot studies are needed to advance EV as a platform for glioblastoma biomarker development.

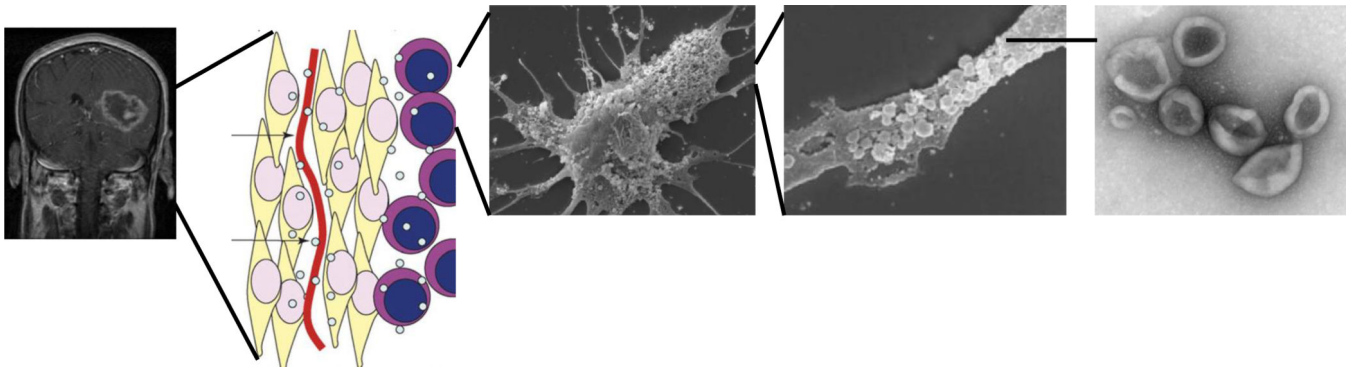

Figure 1. Extracellular vesicles (EV) as a diagnostic biomarker platform

EVs are secreted by glioblastomas and carry tumor-specific genetic materials. **(A)** MRI demonstrating a glioblastoma in the left temporal region. **(B)** The secreted EVs transgress histo-anatomic boundaries of tumor cell (yellow), blood vessel (red) and stroma cell (purple). **(C)** Scanning electron microscopy image of a primary glioblastoma cell. Note the EVs on the cell surface. **(D)** Higher magnification showing the vesicles on the cell surface. **(E)** Electron micrograph of the EVs isolated from the biofluid of glioblastoma patients. Adapted with permission from [18] © Macmillan Publishers Ltd. (2008).