



'Treasure' in garbage bags: extracellular vesicles based biomarker for neurological diseases

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Introduction

Extracellular vesicles (EVs) are micro- to nano-sized membrane-bound vesicles that are secreted by all cell types except viruses as part of their normal physiological function to maintain cellular homeostasis. Initially, EVs were considered as 'garbage bags' that cells utilize to throw their metabolic waste out. However, these vesicles were later recognized to play a crucial role in intercellular communication by transferring various biomolecules (loaded as cargos) such as proteins, nucleic acids (mostly non-coding RNAs), lipids and metabolites. Majorly EVs are divided into three subclasses: apoptotic bodies, microvesicles (MVs), and exosomes. Apoptotic bodies are released from the cells undergoing programmed cell death and range from ~50 to 5 μm in size (1,2). MVs are ~100 to $\geq 1,000$ nm in size and arise by outward budding of the plasma membrane (3). Exosomes are derived from the endosomal system and released to the extracellular environment as ~30–150 nm size vesicles. EVs isolated from *in vivo* system cannot be distinguished if they are generated from the endosomal pathway or released from the outward budding of the plasma membrane. Also, due to their overlapping size and lack of specific biomarkers, it is difficult to discriminate these vesicles and, therefore, collectively referred as EVs. In the last decade, small EVs (sEVs), mainly enriched in exosomes, have garnered much attention for their role as biomarkers for several diseases, including cancer (4,5), cardiovascular disease (6,7), diabetes, and obesity (8,9), inflammation (10) and psychiatric disorders (11,12). The possible isolation of sEVs from almost every biological

fluid and the recent methodological advancement to isolate cell type-specific sEVs pose them as an attractive target for the development of non/less invasive biomarkers for complicated diseases like psychiatric disorders. Analyzing the cargos of neuron-derived EVs (NDEVs) have been shown to predict the development of Alzheimer's disease (AD) up to 10 years before the clinical onset (13). Ledreux *et al.* reported that the NDEVs isolated from the individuals with Down syndrome and AD are loaded with p-Tau and have the tau seeding capacity to spread tau pathology in the mouse brain (14). Recently, our group also reported the utility of NDEVs and other brain cell-derived EVs in identifying the neurodegenerative and pro-inflammatory effects of oxycodone self-administration in cynomolgus monkeys (15).

Does EV size matter?

A recent elegantly designed study by Saeedi *et al.* showed the potential of NDEVs in identifying the effect of an antidepressant drug in major depressive disorder (MDD) patients (16). Authors in that study isolated EVs from the plasma of control and MDD subjects and enriched for NDEVs using neural cell adhesion molecule L1CAM, both before (T0) and after 8 weeks of therapy (T8) with antidepressant drug escitalopram. The characterization of EVs and NDEVs presented some interesting observations, such as; the NDEVs were smaller in size than total EVs and the size of the NDEVs changed as a function of treatment response measured by change (>50% decrease) in

Montgomery-Asberg Depression Rating Scale (MADRS) score. Interestingly, patients responding positively to the therapy showed a significant increase in NDEVs in the size range of 101 to 150 nm, while the concentration of smaller size NDEVs decreased. The authors also confirmed the observation in two separate cell lines (human neural progenitor Rencells and HEK293T cells) as treatment of these cells with the same antidepressant drug increased the size and amount of the EVs. This study proposes that smaller EVs might be loaded with less cargo suggesting a reduction of communication via NDEVs cargo in untreated MDD patients, which is rescued upon escitalopram response.

In the EVs research field, the isolation methodology and EVs characterization are of foremost importance. Use of more than one method for EVs characterization may be opted when drawing any conclusion based on the change in their size and concentration. In this study (16), two methods for EVs characterization were employed, namely, tunable resistive pulse sensing (TRPS) (Izon Science) and nCS1 instrument (Spectradyn). However, both the instruments work on the same resistive pulse sensing technology. It would have been interesting to observe if the reported changes in the size of NDEVs are consistent using a different approach i.e., analyzing light scattering and Brownian motion with nanoparticle tracking analysis or other methods suggested by van der Pol *et al.* (17). Although authors judiciously controlled the TRPS measurements recorded at two different time points by adjusting the measurements at T0 with the mean and standard deviation of the controls at T8.

Interestingly, most of the studies conducted on sEVs to represent them as potential biomarkers for diverse diseases are focused on their cargos. Some of the studies also reported that following chemical or physiological stress amount of EVs secretion increases (18,19). Though most of these studies were performed *in vitro* and any conclusion drawn on the basis of *in vitro* condition may not extrapolate in the *in vivo* system. However, there are not many studies that suggest a significant change in sEVs size following drug treatment and relate that as a function of treatment response. This study by Saeedi *et al.* (16) presented an increase in NDEVs size as a response to antidepressant drug treatment for 8 weeks in the MDD cohort and then confirmed that observation in two cell lines in cell culture. This observation might be reasonable for a drug that can target proteins involved in EV biogenesis. However, given the fact that escitalopram belongs to the selective serotonin

reuptake inhibitors (SSRIs) group of drugs and is not reported to affect the EV biogenesis, the results from this study need to be further pursued to identify the underlying mechanisms. It is also interesting to note that the SSRIs affect the neurons expressing the serotonin transporter, and authors suggested this as a possible hypothesis to explain NDEVs size change; however, the size change in the NDEVs was reported in L1CAM positive NDEVs, which is a general marker for the neurons and not specific to serotonergic neurons or brain neurons.,

The enrichment of cell type-specific EVs from plasma has provided an immense possibility to predict the pathophysiological state of the parent cell, especially in deeper tissues or for hard-to-access organs. However, one should be cautious while selecting the marker protein to isolate a specific EV subpopulation. Using two or more surface markers could be a better approach to confirm the changes in cell- or tissue-specific EVs. The selected markers could either be used sequentially to isolate cell type-specific EVs, or the effect observed with the selected first marker used for EVs isolation could be verified using a second surface marker. In the study by Saeedi *et al.* (16), NDEVs were enriched using neuronal marker L1CAM, and then a few of the key observations were further verified using another neuronal marker synaptosome-associated protein 25 (SNAP25) in an independent cohort. This is certainly convincing considering a recent conflicting report on L1CAM presence on EVs in plasma or CSF (20), though it is widely reported to enrich NDEVs. Compared to L1CAM, SNAP25 expression is mainly limited to brain tissues, and it will be interesting to see the relative abundance of SNAP25+ EVs in plasma and their utility in characterizing biomarkers for neurological and psychiatric diseases.

MicroRNAs (miRNAs) cargos in EV

Analyzing the cargos of EVs seems more reasonable as those could possibly reflect the pathophysiological state of the parent cells. Many studies have shown that analyzing the EV cargos (mainly proteins and miRNAs) can predict the onset, progression, and treatment response of psychological disorders (13,15,21). The pathological proteins associated with neurodegenerative diseases like α -synuclein, p-tau/tau, and amyloid β could be transported through EVs to nonpathological areas promoting the progression of disease (22,23). miRNAs loaded in EVs are relatively more stable compared to free circulatory miRNAs and are also involved

in the pathogenesis of neurodegenerative and psychiatric disorders (12,24). Characterizing the miRNAs loaded in EVs could serve as a signature to represent the host cell's status. Though it is also important to consider that the circulatory miRNAs are not always packed in EVs, they can freely circulate in the biofluids. While analyzing the miRNAs in EVs is important and informative, great care should be taken to isolate EVs from the biofluids as it is important to remove miRNAs that might be arbitrarily sticking to the EVs membrane. In the study by Saeedi *et al.* (16), the isolated NDEVs were treated with RNase A so that the analyzed miRNAs will represent the tissue of origin and not some random miRNAs non-specifically sticking to the EVs surface. The specificity of the analyzed miRNA expression was further confirmed by comparing them with the miRNAs isolated from the brain tissue-derived EVs. The study showed that the analyzed miRNAs in the NDEVs of MDD patients actually mirror the EV miRNAs extracted from brain tissue with 89% overlap in the number of identified miRNAs. Interestingly, these miRNAs identified in NDEVs were shown to represent the tissue identity as they did not overlap at all with the miRNAs reported from the saliva EVs (25).

Furthermore, analyzing the miRNAs cargos of the NDEVs isolated from controls and MDD patients, both before and after 8 weeks of therapy, authors identified 75 miRNAs using small RNA sequencing analysis that showed at least three counts in 70% of a given group (control, responders and non-responders). The authors compared if the expression of these miRNAs altered with the treatment response. The analysis identified 9 miRNAs (miR-423-3p, miR-191-5p, miR-486-5p, miR-30d-5p, miR-425-5p, miR-25-3p, miR-21-5p, miR-335-5p, and miR-126-5p) changed from before to after therapy with statistical significance based on small RNA sequencing data. The backward stepwise regression analysis was performed to determine if any combination of these miRNAs can predict the response of the therapy ('responders' vs. 'non-responders'). The authors identified the combination of three miRNAs miR-21-5p, miR-30d-5p, and miR-486-5p changed over therapy and can predict the treatment response. However, the direction of change of these miRNAs was not reported (increase or decrease with therapy). Also, the change in expression of these three miRNAs was validated using qPCR, and expression of only miR-21-5p showed a significant correlation with the sequencing data. One important thing to note while using qPCR to validate the sequencing data is the selection of normalizing control.

The authors in this study used miR-103a-3p and miR-9-5p as normalizing control to calculate the relative fold change ($2^{-\Delta\Delta C_t}$). The selection of miR-103a-3p seemed reasonable as it was based on its stability as assessed using NormFinder. Although miR-9-5p displays a conserved expression pattern in the central nervous system, its expression in the brain is reported to be altered with stress, and it is involved in several neurodegenerative diseases (26). The selection of stable miRNA that can be used as normalizing control for the qPCR data is very context-dependent as miRNAs expression, especially in the EVs, can change in different disease conditions. It will certainly not be equitable to select one miRNA as a universal normalizing control even if it shows conserved expression in a particular tissue. The selection of miRNA should be based on its stability in that particular cohort in a given disease context. Alternatively, if it is difficult to identify the stable miRNA for normalization of the data, external miRNA control (spike-in control) can be used. *Caenorhabditis elegans* miRNA-39-3p (cel-miR-39-3p) can be used for this purpose and can be mixed with EVs before the isolation of RNA. Though it mainly serves as a control for the efficiency of RNA isolation or cDNA synthesis, in certain conditions it can also be used as a normalizing control.

Validation is important

It is also important to cogitate if the observed change in the expression of miRNAs is consistent and does not change with the choice of marker used to isolate NDEVs. To confirm the expression of miRNAs observed in L1CAM positive EVs, authors in this study used a second marker SNAP25 to isolate NDEVs from a separate validation cohort and showed the consistency in the expression of two miRNAs (miR-486-5p and miR-30-5p), out of three miRNAs (miR-21-5p showed no change) used as a combination to predict 'responders' vs. 'non-responders'. However, the ratio of change on these two miRNAs at T8/T0 in responders is close to one, which suggests that their expression did not change over time in the patients who responded to therapy, though it decreases (less than one) in non-responders. Since the change in miRNA expression in NDEVs represents the brain status, it is important to relate their functional consequences and involvement in treatment response (or disease progression). Enrichment of target prediction of these three miRNAs showed that their targets are enriched in the brain tissue. This corroborates the authors' other findings in this study that the miRNAs

identified in NDEVs significantly overlap with the miRNAs from brain tissue-derived EVs.

Clinical significance

The analysis of cargos of EVs (or cell type-specific EVs) is certainly exciting; however, whether those could be of any clinical significance or provide any translational application is also critically important. If the cargos of EVs can distinguish between ‘control’ or ‘diseased’ subjects or treatment ‘responders’ vs. ‘non-responders’, it will be of high translational significance. The comparable changes in the expression of targets of identified miRNAs in this study with the brain tissue (anterior cingulate cortex) of individuals who died by suicide during an episode of major depression represent the high clinical prominence of NDEV miRNAs. Interestingly, the mRNA sequencing of the brain tissue of control and those who died by suicide show differential expression, which overlaps with the identified mRNA targets of miRNAs loaded in NDEVs. Though the data presented in this study compared the subjects before and after the therapy, it will be very interesting to see if it is possible to identify the patients who may respond to therapy by analyzing the NDEVs’ cargo (or possibly size) at the start of the therapy.

Conclusions

In conclusion, Saeedi and colleagues (16) have presented an exciting study to show the utility of NDEVs to identify the response to antidepressant therapy. They also presented an interesting observation that the size of the NDEVs is smaller compared to total EVs, and NDEVs size is modulated as a response to therapy. The use of more than one marker to isolate NDEVs (L1CAM and SNPA25) in this study is also a step in the right direction. It will be interesting to see whether NDEVs isolated using these two markers are overlapping or distinct. Most importantly, miRNA analysis of NDEVs and their targets show a similar change in the brain tissue of the patients who died by suicide during MDD. This suggests that analysis of miRNA cargos in plasma NDEVs could serve as a proxy marker to predict the pathophysiological status of the brain cells and treatment response. These findings and approaches are certainly applicable to other neurological and psychiatric disorders.

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Footnote

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