Profiling of plasma-derived extracellular vesicles cargo for diagnosis of pancreatic malignancy

Theresa L. Whiteside[1,2]

[1]Departments of Pathology, Immunology and Otolaryngology, School of Medicine, [2]UPMC Hillman Cancer Center, University of Pittsburgh, Pittsburgh, PA, USA

Correspondence to: Theresa L. Whiteside. 5117 Centre Avenue, Suite 1.27, Pittsburgh, PA 15213, USA. Email: whitesidetl@upmc.edu.

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Extracellular vesicles (EVs) and their smallest subset, exosomes, have been emerging as potentially promising biomarkers of cancer detection, prognosis and response to therapy. There are several reasons why EVs, and especially tumor-derived exosomes, have become of great interest as "liquid biopsies" to investigators engaged in monitoring cancer progression and treatments. Tumors produce a variety of EVs (exosomes, microvesicles, apoptotic bodies, collectively called "tEVs"), all of which carry tumor-derived molecular and genetic materials (1). However, the cellular mechanisms responsible for tEVs formation and release by the parent cell differ for different EV types (2). While microvesicles "pinch off" from the cell surface, exosomes originate from the endocytic compartment of the parent cell and are released into intercellular space upon fusion of the multivesicular bodies (MVBs) with the surface membrane. Apoptotic bodies are large aggregates of dying cells.

Because of their unique biogenesis, tumor-derived exosomes or "TEX" carry protein and mRNA profiles that highly reflect contents of the parent tumor cells. For this reason, exosomes (30–150 nm) rather than larger microvesicles (200–500 nm MVs) are considered to be more reliable "liquid biopsies." Although tumors are known to produce tEVs in an excess and disseminate them throughout all body fluids and tissues, the ratios of tEVs to total circulating EVs in plasma likely vary from one cancer patient to another, depending on the tumor size, stage and activity as well as exosome clearance from the circulation. Interestingly, recent evidence suggests that exosomes carry CD47, a "don't eat me" protein, which retards their clearance from the circulation (3) and increases their importance as circulating biomarkers. Thus, tEVs appear to be most attractive among EVs as "liquid biopsies." However, major barriers to their clinical use exist. The complexity of EV isolation methods from body fluids and tEV separation from other, non-tumor cell-derived, EVs in patients' plasma are the two major obstacles. The current lack of a precise nomenclature that would distinguish exosomes from MVs is another difficulty (4). High-speed ultracentrifugation conventionally used for EV isolation is lengthy, cumbersome and inefficient (5). TEX separation from non-TEX is based on the recognition of specific cancer markers TEX are expected to carry, yet with the exception of mutated proteins, few such markers are available.

To overcome these existing barriers and evaluate the role of TEX as "liquid biopsies," methods are needed that would dispense with ultracentrifugation for EV isolation, utilize antibodies (Abs) specific for tumor antigens TEX carry to enable their capture and usher ultrasensitive assay systems for detection of cancer markers on the captured TEX. A recent paper by Yang *et al.* published in *Science Translational Medicine* (6) describes a novel multiparametric profiling system incorporating arrays of nanoplasmonic sensors (NPS) for capture of tEVs and detection of their cargo components. This system is specifically designed

for rapid clinical workflows, small clinical samples and high-throughput Ab-based capture and detection of tEVs in plasma of patients undergoing surgery for pancreatic pathologies.

The NPS microarray or chip the authors developed contains a series of nanopores (200 nm in diameter) periodically spaced in a 100 nm-thick gold film which is coated with neutravidin. The pores are charged with biotinylated tumor antigen-reactive Abs which capture tEVs <200 nm in diameter, binding them to pores and inducing a spectral shift of light transmitted through the nanopores to red. This red shift is detected by sensors and reflects the amount of tEVs captured in the Ab-charged nanopores. The number of sensing sites in the array can be up-scaled to as many as 1,000, but the authors of this paper worked with 100 detection sites and 25 different Abs used in quadruplicate. Molecular printing methods were used to print Abs and exosomes on the sensor chip. Transmission spectra were scanned, collected and analyzed. Printing and measurements were automatically operated and optimized for processing of clinical samples. The array system, utilizing 200 nm pores, appears to be designed to capture exosomes and, indeed, the TEM images of vesicles bound to the pores indicate they are <100 nm in diameter. Nevertheless, the authors chose to call the captured vesicles "tEVs."

The authors first tested and calibrated the NPS system using tEVs derived by ultracentrifugation from supernatants of pancreatic cancer cell lines and PDX cell lines, so that all or most of isolated vesicles placed on the arrays were tumor-derived. The 15 Abs selected for capture of these tEVs were first shown to be reactive with antigens expressed by PDAC cell lines and found on EVs produced by these cell lines. These initial studies were done with 15 putative cancer cell markers which included EGFR, EPCAM, HER2, MUC1, GPC1, WNT2, GRP94 as well as EV markers CD63, RAB5B and CD9. The results showed good association (r=0.86) between expression profiles seen in whole cells and tEVs. Next, tEVs were obtained by ultracentrifugation from plasma of 22 PDAC patients and 10 healthy donors and tested for 4 pan-cancer markers (EGFR, EPCAM, HER2 and MUC1) and three putative PDAC markers (GPC1, WNT2, GRP94) individually and together. While no single marker achieved sufficient sensitivity or specificity, the panel of markers including EGFR, EPCAM, MUC1, GPC1 and WNT2 (named "the $PDAC^{EV}$ signature") showed improved specificity and sensitivity with accuracy of 100% in distinguishing PDAC from healthy donors in this training cohort.

The validation data for the $PDAC^{EV}$ signature were obtained using plasma obtained from a prospective cohort of 43 patients undergoing surgery for pancreatic cancer (n=35) or other abdominal indications (n=8). Tissues were available for pathologic diagnoses of 22 PDAC patients; 8 patients with pancreatitis and 5 with benign cystic tumors. The heat map analyzes showed that no single patient had elevations of similar markers, no single marker differentiated patients from controls and only the combination of markers comprising the $PDAC^{EV}$ signature differentiated PDAC patients from those with benign conditions and controls with an accuracy of 84%, sensitivity of 86%, and specificity of 81%. The $PDAC^{EV}$ signature measured in the multiparametric NPS assays had higher sensitivity, specificity and accuracy than the clinical gold standard assays for PDAC serum biomarkers, CA19-9 and CEA.

The overall message conveyed by the authors of this well designed and expertly performed biomarker study is that the NPS platform designed to capture tEVs from patients' plasma and to perform automated multiparameter profiling of tEV components offers a clinically feasible, high throughput, low cost "liquid biopsy" assessment in which tEVs serve as surrogates of the tumor.

The study, while confirming the role of tEVs as potentially clinically useful biomarkers of PDAC, falls short of validating tEVs as "liquid biopsies" of PDAC. The authors had an opportunity to compare the $PDAC^{EV}$ signature of tEVs that was defined in the training set with that of surgically removed patients' tumors in the validation cohort. This opportunity was not realized, and the $PDAC^{EV}$ profile was only used to compare tEV cargos of cancer *vs.* benign patients and *vs.* healthy donors. Yet, until it is demonstrated by direct comparisons that circulating tEVs and the parent tumor tissues have the same protein profiles, the concept of "liquid biopsy" remains questionable.

Further, the origin and nature of vesicles captured on the arrays remains unclear. Their definition as tEVs is based on a panel of Abs that are tumor antigen-reactive not tumor antigen-specific. Thus, any vesicle in plasma <200 nm in diameter, whether tumor- or normal cell-derived, that is "seen" by the Abs included in the panel is captured, reducing specificity of detection. The authors' conclusion that only a panel of tumor-associated antigens carried by tEVs and not a single antigen, such as e.g., GPC1, is useful for discrimination of PDAC from benign or normal conditions is likely correct. However, this is because none

of the Abs used for capture of tEVs were specific for PDAC. The antigens detected by these Abs are overexpressed by the tumor but are also expressed by a variety of normal cells. The Ab-based capture, as used here, has led to enrichment in exosomes which were likely a mix of tEVs and normal cell-derived EVs. Hence, the specificity and sensitivity of the capture was in the 80% range. It is likely that the use of a tumor-specific capture Ab, were it available, could lead to the highly specific isolation of PDAC-derived exosomes, similar to what Melo *et al.* reported with their GLPC1-based capture of TEX (7). In the absence of highly tumor antigen -specific high-affinity capture Abs, the use of the panel of non-specific Abs to antigens overexpressed on tumor cells is the second best. Perhaps this is as good as can be currently expected. It might be possible to improve capture of tEVs in the future by the development of a new genre of mAbs specific for epitopes present only on tumor cells of one histologic tumor type but not on normal cells.

While the described NPS detection technology for the tEVs cargo is high throughput, clinically applicable and affordable, it is used here to immune capture tEVs using total EVs isolated from plasma by ultracentrifugation. This imposes a significant limitation to its routine clinical application. Harvesting of EVs from plasma by ultracentrifugation, currently a widely used method, is not practical for clinical monitoring. Other technologies for EV isolation from body fluids, including, e.g., size exclusion chromatography (8), are rapidly emerging that might provide a more effective and practical approach to the acquisition of EVs for capture. However, any two-step strategy, especially one including a cumbersome sample preparation required for testing, has limited clinical usefulness. Improvements would include the development of a strategy that captures tEVs directly from plasma of PDAC patients on the NPS arrays and measures their molecular content with the same degree of specificity and sensitivity as those seen with isolated EVs. Only then could the NPS measurements be seamlessly applied to longitudinal profiling of tEVs in large patient cohorts as visualized by the authors of this paper. It is likely that improvements in application of the NPS platform to measurements of tEVs in plasma will soon materialize allowing for its translation to the clinical arena.

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Footnote

Conflicts of Interest: The author has no conflicts of interest to declare.

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