Editorial Commentary

# The potential of microRNA carried by small extracellular vesicles in cancer plasma to serve as cancer biomarkers

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Small extracellular vesicles (sEV) aka exosomes are intercellular communication vehicles that are produced by all cells and circulate freely, delivering messages to closely or distantly located cells (1). Exosomes carry and distribute a broad variety of proteins, lipids, glycans and nucleic acids, some located in the vesicle lumen, others presented on the vesicle surface membrane to recipient cells (2). During an interaction with recipient cells, sEV deliver signals that lead to their uptake via mechanisms, such as receptor/ligand interactions, membrane fusion, endocytosis or phagocytosis, that facilitate their entry into the cytosol (3). Here, the vesicles disrobe, and their molecular content becomes incorporated into the cellular machinery, resulting in the phenotypic and functional reprogramming of recipient cells (4). In this context, the sEV produced by tumor cells have been of special interest. Because their molecular and genetic content recapitulates that of the parent tumor cells, circulating tumor-derived sEV are viewed as liquid surrogates of parental tumor cells and could potentially serve as liquid tumor biopsy. Currently, the clinical significance of sEV, and specifically the role of protein and nucleic acid cargos they carry, in diagnosis and prognosis of cancer is intensively investigated.

Numerous reports have focused on microRNA (miRNA) secreted by tumor cells via sEV (exo-miRNA) as potential biomarkers of cancer. A recent report by Galiveti *et al.* (5) provides an example of current efforts designed to establish the translational potential of exo-miRNA as liquid biopsy for early detection of head and neck squamous cell carcinoma (HNSCC). Among human solid tumors, HNSCC has a relatively poor prognosis with an overall 5-year survival of about 50–60%. Most patients with HNSCC present with advanced stage disease, and early detection by visual screening and palpation lacks sensitivity. There is an urgent unmet need for the discovery and development of novel biomarkers to improve screening and early detection of HNSCC.

The emergence of exo-miRNA as a potential noninvasive biomarker of HNSCC was initially based on the studies of sEV isolated from supernatants of HPV(+) and HPV(–) HNSCC cell lines (6-8). Since the HPV(+) and HPV(–) HNSCCs differ in the incidence, clinicopathologic features, response to cancer therapy and prognosis (9), it was expected that sEV these cell lines produce might have different characteristics. To test this hypothesis, miRNAs in sEV secreted by cultured HPV(+) *vs.* HPV(–) tumor cells were analyzed, searching for differences in exo-miRNA profiles. Indeed, as shown by Galiveti *et al.* (5) and other relevant studies (7,8), comparisons of exo-miRNA isolated from supernatants of various HNSCC cell lines clustered the identified exo-miRNA according to HPV status. Marked differences in exo-miRNA profiles between HPV(+) and HPV(–) cells were found, although there was a considerable degree of overlap. High numbers of differentially expressed

exo-miRNA transcripts were identified in these comparisons, and differentially expressed miRNA species present in sEV differed among various studies (5,7). Importantly, a comparison of the exo-miRNA profiles in sEV from HNSCC cells with sEV isolated from non-pathologic oral epithelial control cells discriminated cancer from non-cancer in Galiveti's study (5). These *in vitro* studies using HNSCC cell lines as a source of tumor derived sEV demonstrated that: (I) the isolated sEV yielded sufficient quantity and quality of mRNA for miRNA seq, providing adequate sequencing reads for the detection of multiple differentially expressed miR transcripts; (II) exo-miRNA recapitulated the miR content of parental tumor cells (7); (III) the identified exo-miRNA profiles differentiated HPV(+) from HPV(–) tumor cells (5,7); and (IV) differentially expressed exo-miRNA discriminated tumor cells established from primary *vs.* recurrent HNSCC (5). In aggregate, these *in vitro* studies illustrated a strong translational potential of exo-miRNAs as HNSCC biomarkers, despite the diversity of methods used for sEV isolation and their profiling.

The objective of Galiveti *et al.* study (5) was to evaluate the potential of exo-miRNAs from patients with HNSCC as biomarkers of early-stage disease. To this end, sEV were isolated from body fluids collected prior to therapy from patients diagnosed with early stage HNSCC [American Joint Committee on Cancer (AJCC) stage I/II]. In principle, saliva, serum, or plasma can be used for sEV isolation (10). While all sEV in supernatants of cultured tumor cells are tumor derived, body fluids contain a heterogenous mix of vesicles differing in cellular origin, size, and cargos they carry (10). Tumor-derived sEV are a subset of total vesicles present in body fluids of cancer patients and may be variably enriched in progressive disease (10). Using archival serum from 22 early stage HNSCC patients and from 10 non-cancer controls, whose demographic characteristics marched those of HNSCC patients, Galiveti *et al.* isolated sEV by differential ultracentrifugation which included a 30% sucrose cushion (5). The characteristics of these sEV as determined by nanoparticle tracking analysis (NTA), transmission electron microscopy (TEM) and Western blots (WBs) for TSG101 and CD81 were consistent with their designation as sEV, although the cellular origin of these sEV varied broadly, and only an unspecified percent of vesicles was tumor derived. Total RNA isolation followed by miRNA seq identified 41 mature differentially expressed exo-miRNAs (24 were overexpressed and 17 underexpressed) in early stage HNSCC cases relative to exo-miRNA in non-cancer controls. The four most highly overexpressed exo-miRs: 3168, 451a, 16-2-3p and 125a-5p were significantly elevated in sEV of HNSCC cases relative to controls. Interestingly, exo-miR-451a and exo-miR-16-2-3p were significantly overexpressed in sEV from all 8 and 7/8 HNSCC cell lines, respectively, relative to sEV from non-malignant cell lines. This appeared to be independent of HPV status, since four of the HNSCC lines were HPV(–) and the other four were HPV(+). No significant correlations were seen between the four top exo-miRNAs overexpressed in early stage HNSCC and key clinical and demographic characteristics in HNSCC patients, except for exo-miR-451a, which only weakly correlated with the primary tumor site. Also, only exo-miR-125a of the four exo-miRs found overexpressed in early HNSCC by Galiveti *et al.*, was identified as significant in two other studies of secreted exo-miRNAs in HNSCC reported in the literature (11,12). This suggests that changes in exo-miRNA expression levels seen in HNSCC cancer might be more difficult to correlate with disease status than initially expected for reasons that are only speculative at this time.

sEV in body fluids of cancer patients are being evaluated as potentially informative elements in liquid tumor biopsy (13). It should be noted that in most cases, it is the sEV protein profiles that are considered as candidate biomarkers of early diagnosis, prognosis, or response to therapy in cancer. The role of circulating exo-miRNA as cancer biomarkers remains speculative, although literature abounds with reports on dysregulated miRs in tumor-derived sEV in cancer plasma/serum. The question arises why there is discordance between sEV carrying proteins *vs.* the same sEV carrying miRNA as potential cancer biomarkers?

One of the major difficulties in the interpretation of exo-miRNA dysregulated expression levels in cancer is the complexity resulting from the ability of each miRNA detected in sEV to target multiple mRNAs. For example, for exo-miR 451a, 27 experimentally confirmed mRNA targets were listed in the database, including multiple genes in various signaling pathways. For exo-miR 16-2-3p, there are 2,886 experimentally confirmed targets in the database. Further, both these miRs and miR-125a-5p are overexpressed in a variety of different solid tumors and associate with numerous cancer-associated pathways. In this context, the putative value of differentially expressed exo-miRNA in patients' sera as biomarkers of HNSCC or of early stage HNSCC is not supported by the data presented by Galiveti *et al.* (5). Instead, it appears that the differentially expressed exo-miRNA in sera and in supernatants of HNSCC cell lines accurately discriminate cancer from

 

non-cancer and thus fit in the category of general cancer biomarkers. Given that cancer development and progression involve genetic changes, the release by tumor cells of sEV carrying dysregulated miRs may well be a sign of malignancy, providing a "yes" or "no" answer. But to establish the value of the profile of differentially expressed exo-miRNA, or even of an individual differentially overexpressed exo-miR, as predictive or prognostic biomarkers in cancer requires further exploration of molecular events in recipient cells which are specifically impacted by the differentially expressed exo-miRs. This requires cloning of the selected dysregulated miR into a relevant recipient cell and linking its presence to a molecular target and the function(s) this target has in carcinogenesis. Hence, various reports in the literature claiming a biomarker role for an exo-miR solely based on its differential expression in a body fluid without downstream functional studies and clinical confirmation should be taken with great caution. This level of complexity does not exist in studies of protein cargos in sEV, where stable profiles of a few overexpressed proteins specify not only the cancer presence but also discriminate tumor types and quantify changes occurring during tumor progression/regression (14).

Perhaps we should not be surprised by reports of multiple dysregulated exo-miRNA in body fluids of patients with cancer. As pointed out by Galiveti *et al.*, exo-miRNA are highly dysregulated in HNSCC and other cancers. Cancer plasma contains millions of sEV, most of them derived from tissue cells reprogrammed by the tumor to contribute to cancer progression and containing variously dysregulated miRs. To expect that a profile of dysregulated exo-miRNA characterizing, e.g., HNSCC but not any other tumor type, can be identified, remains stable during cancer progression/regression, or predicts response to cancer therapy is probably unrealistic. While Galiveti *et al.* remain optimistic about the future of exo-miRNA as early detection biomarkers for HNSCC, where a "yes" *vs.* "no" decision replacing palpation or visual examination may be an option, the usefulness of exo-miRNA in diagnosis and prognosis of other types of cancer remains questionable. The concern is that with so many elements in the puzzle (i.e., numerous miRs in every vesicle and numerous vesicles with multiple miRs originating in different cells) that simultaneously change in opposite directions, the hope of finding a stable prognostic exo-miRNA profile is not encouraging. Thus, numerous combinations of variously dysregulated exo-miRNAs might emerge that flag the presence of cancer. However, without serial sampling of sEV carrying the flag to denote time of their appearance or a change in their miR profile as cancer develops, they are not likely to qualify as biomarkers of early/late disease. Their predictive or prognostic value in disease is also questionable, given the large number of mRNA targets for each miRNA and a lack of compelling evidence for the exo-miRNA profile that discriminates different tumor types or undergoes predicted changes during therapy.

The hope is that as we learn more about the sEV biology, their packaging and release from parent cells as well as their uptake and entry into recipient cells, some of the puzzles related to cargos they carry and deliver to recipient cells will be solved. For example, exo-miRNA profiles that differentiated HPV(+) from HPV(–) tumor cell lines were identified in several different studies (5,7,8), suggesting that the sEV derived from tumor cells are more likely to qualify as cancer biomarkers than sEV isolated from body fluids, which contain a mix of vesicles with different cellular origins. As methods for isolation of tumor-derived sEV (TEX) from cancer plasma are becoming available (10), the search for informative exo-miRNA profiles in TEX might come to fruition in the near future. Current literature provides evidence that exo-miRNA secreted by different cells might have different biological roles due to their packaging specificity (15). If so, then focusing on miRNA carried by TEX and their functions in the tumor microenvironment may provide an opportunity for evaluating the exo-miRNA potential as cancer biomarkers.

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aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.



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