

Circulating tumor cells and extracellular vesicles as liquid biopsy markers in neuro-oncology: prospects and limitations

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Abstract

For many tumor entities, tumor biology and response to therapy are reflected by components that can be detected and captured in the blood stream. The so called “liquid biopsy” has been stratified over time into the analysis of circulating tumor cells (CTC), extracellular vesicles (EVs), and free circulating components such as cell-free nucleic acids or proteins. In neuro-oncology, two distinct areas need to be distinguished, intrinsic brain tumors and tumors metastatic to the brain. For intrinsic brain tumors, specifically glioblastoma, CTCs although present in low abundance, contain highly relevant, yet likely incomplete biological information for the whole tumor. For brain metastases, CTCs can have clinical relevance for patients especially with oligometastatic disease and brain metastasis in cancers like breast and lung cancer. EVs shed from the tumor cells and the tumor environment provide complementary information. Sensitive technologies have become available that are able to detect both, CTCs and EVs in the peripheral blood of patients with intrinsic and metastatic brain tumors despite the blood brain barrier. In reference to glioblastoma EVs, being shed by tumor cells and microenvironment and being more diffusible than CTCs may yield a more complete reflection of the whole tumor compared to low-abundance CTCs representing only a fraction of the multiclonal tumor heterogeneity. We here review the emerging aspects of CTCs and EVs as liquid biopsy biomarkers in neuro-oncology.

Keywords

brain metastasis | glioblastoma

The concept of capturing circulating biomarkers as “liquid biopsy” (LB) has been first introduced for CTCs by Pantel and Alix-Panabieres (Trends Mol Med. 2010) and then rapidly adapted to other circulating analytes such as cell-free nucleic acids or EVs. LB has been recently highlighted as one of the milestone discoveries of the past two decades (Nature Milestones December 2020). For glioblastoma (GBM), the concept of LB has been extrapolated from other tumor entities despite the fact that shedding of cells or cellular products might be hindered by the blood brain barrier. The original main interest for circulating biomarkers in extracranial cancer was focused on CTCs and was

aimed at the detection of minimal residual disease and monitoring of oncological therapies.¹ CTCs are an indicator for manifest or imminent metastatic disease and mostly responsible for tumor spread apart from lymphatics. CTCs thus are undoubtedly the cause for brain metastasis from systemic cancer but for glioblastoma, there is no consequence of CTCs reflected in the typical disease course. As glioblastoma (GBM) is a highly vascularized tumor with an anarchic vascular architecture of massive vessel sprouting and intrinsic thrombosis, it seemed only consequential to this, that there should also be CTCs in GBM. However, except for anecdotal cases,² regular occurrence of

systemic metastases has not been observed despite these tumors often being manifold the size of an original melanoma or breast cancer having already caused wide spread dissemination. Arguing, that the disease course is in most cases very rapid and metastases will not appear in such short time,³ one should then sequentially expect occasional extracranial metastasis at a higher rate in long-term survivors, who will live three years or longer. That support, however, is lacking. Proof of concept was only obtained from several cases of GBM originating in transplanted organs in immunosuppressed patients obtained from donors, suffering from GBM.² This observation points to the hypothesis that immune surveillance or other microenvironmental conditions might prevent CTCs to form an overt metastasis at extracranial sites and allows the assumption that CTCs from GBM may be more frequent than assumed.

CTCs, GBM

Despite initial (wrong)-negative reports to find GFAP-expressing cells using PCR for the GFAP transcript in plasma⁴ continuing systematic studies using other methods to search for CTCs in GBM patients were performed and the uniform result has been, that they exist in a considerable percentage of patients but in very low abundance. Not being able to use EpCam and Keratin which are the standard markers for CTC enrichment and detection in epithelial cancers^{1,5} different technologies, using a broad array of enrichment techniques, centrifugation paradigms and subsequent detection systems based on GFAP immunostaining and EGF-R gene amplification,⁶ telomerase activity assay⁷ or antibody cocktails (STEAM: Sox 2, tubulin beta 3, EGFR, A2B5, and c-MET⁸) lead to detection rates of 20 to 50% of GBM patients. Also physical properties like size and elasticity have been reported more recently in microfluidic devices, interestingly again using GFAP as a marker and cell surface vimentin based on the finding that CTCs from GBM show a mesenchymal phenotype⁹ but validation of broad applicability is pending. Uniform to all reports is the low yield of mostly only few CTCs per blood sample of 5–10 ml and for lack of definitive uniform markers for GBM derived cells, additional proof of the malignant identity, genomic analysis on the single cell level by FISH, CGH or exome sequencing are needed.^{6,10}

There are no reliable clinical correlations so far between patients negative or positive for CTCs. In our cohort of 126 patients, we saw no correlation of the detection of CTCs with survival,⁶ no correlation with tumor-infiltrating CD68-positive cells (microglia/macrophages) but finally, a statistically significant decrease of CD3- and CD8-positive T-cell infiltrates in the corresponding tumor tissues.¹¹ There have been other approaches to isolate and possibly expand patient isolated cells for further analysis by culturing them for a few days after transfection with telomerase conditionally replicating adenovirus for later identification. This approach allowed for the detection of Wnt pathway-associated stemness characteristics in these patient-derived CTCs in subsequent animal studies.¹² We have been unsuccessful to cultivate CTCs from GBM although that would be an extremely interesting population to study

but probably unattainable. It requires an abundance of CTCs like in the establishment of primary CTC cell cultures in other tumor entities like breast or colon cancer where in rare cases several thousand CTCs were captured.^{13,14}

Ensuing from the above, the practical relevance of GBM derived CTCs and their guidance potential also for glioblastoma management is still unresolved, especially their potential to track the progress or failure of therapy and reliably distinguish between true progression and pseudoprogression, a supposedly central issue which, however, can be challenged for its real practical value. In addition, there is the issue of how representative CTCs from GBM are for any given tumor as most likely they only represent one or few subclones from areas of completely broken down BBB while it is unlikely that cells escape the leading edge of the tumor or the infiltrated areas stretching far throughout the affected hemisphere or even the contralateral side.¹⁵ CTC “clusters” which have been reported recently with a microfluidic device would even be less representative of the infiltrative, invasive edge, and also should have a higher propensity than single CTCs to form metastases as suggested for other entities like breast cancer^{10,16} but for whatever reason not seen in GBM patients. Therefore this issue still needs more supportive evidence with more stringent detection protocols.

With their use as biomarker limited by abundance and lack of representation of the tumor complexity, CTCs in the GBM context are nevertheless an interesting subject to study in their own right and an extremely important part of the puzzle to solve the biology of GBM. They may help to understand the specific requirements (seed soil) and immunological checkpoints which seem to prevent distant metastases. In the context of studies in animal models, it is for example unresolved whether those rare CTCs, or CTC-clusters could have an impact on cerebral dissemination. In a mouse model, stemness of CTCs has been shown¹² as well as a homing capacity to the original tumor site. Although it is a matter of fact that single glioblastoma cells will have disseminated throughout the brain once a tumor becomes manifest, these homing data raise the potential of the role of CTCs also in hematogenic spread/local recurrence of CNS tumors as it is already established for medulloblastoma in parabiotic animal studies.¹⁷ If glioblastoma CTCs would indeed have a high degree of stemness, as must be suspected from the typical glioblastoma histology in the tumors arising from circulating cells in transplant patients, they would be excellent candidates to seed distant recurrences or multifocal tumors in the permissive brain environment. They may even after radiologically complete resection re-enter the peritumoral region from the extracranial compartment, where undoubtedly they are present, have stemness properties and mesenchymal characteristics, and may be dormant for lack of hospitable microenvironmental surroundings. Such hypotheses are difficult to prove in the human situation but seem to gain substance in animal models.¹² In the most recent study in which the microfluidic device was used to isolate CTCs, in the small number of patients with high numbers of CTCs, survival was the shortest.⁹ It is obvious, that there will be much to learn from continuous studies on CTCs from GBM although probably not for their potential as therapy-monitoring biomarkers.

CTCs, Brain Metastases

Brain metastases (BM) are a rapidly expanding area in neuro-oncology due to the increasing incidence rates of brain metastases especially in breast cancer and melanoma patients due to much improved extracranial disease control.¹⁸ Therefore, it is only consequential, that the role for CTCs is explored in this context as they would be the only way, how the brain metastasis initiating cells (BMICs)¹⁹ can get to the brain. Being a very special, well-protected environment, it appears that defined criteria need to be fulfilled for cells to thrive in this environment. So what has been named “competence” is needed for different tissue origins like breast or lung to get to the brain and this has been ascribed to the CTCs.^{19–22} Animal studies suggest, that these BMICs apparently originate from a small subpopulation of slow-cycling cells characterized by NDRG1 expression which is corroborated by correlative studies in a human breast cancer patient cohort.¹⁹ Another signature was found in a xenograft model generated from EpCAM negative breast cancer CTCs which were found to express HER2, EGFR, HPSE, and Notch1 and effectively generated brain metastases.²¹ In general, similarly to GBM, a more mesenchymal phenotype of CTCs has been described for patients with brain metastases.^{20,23} Therefore, much used EpCAM based CTC detection methods, such as the FDA cleared CellSearch system, are perhaps not optimal when analyzing samples from brain metastasis patients. Still, although fewer CTC are detected, these are clearly associated with worse prognosis of patients with brain metastasis.^{24,25} While the signatures for BMICs seem to vary between different tissue origins, loss of PTEN, copy number variations with gains of chromosome 7, EGFR amplification, and over-expression of other HER-family members (HER2 and HER3) seem to have a strong association with BMs^{26–30} and that pattern is surprisingly similar to the aberrations found in glioblastoma, thus seemingly conveying a tissue/“soil” preference for these CTC clones. Furthermore, we and others have shown that those CTCs that seem to be able to transmigrate through the BBR express specific proteins such as semaphorin 4D, ALCAM, and CD74 facilitating the trans-endothelial migration into brain parenchyma.^{31–33}

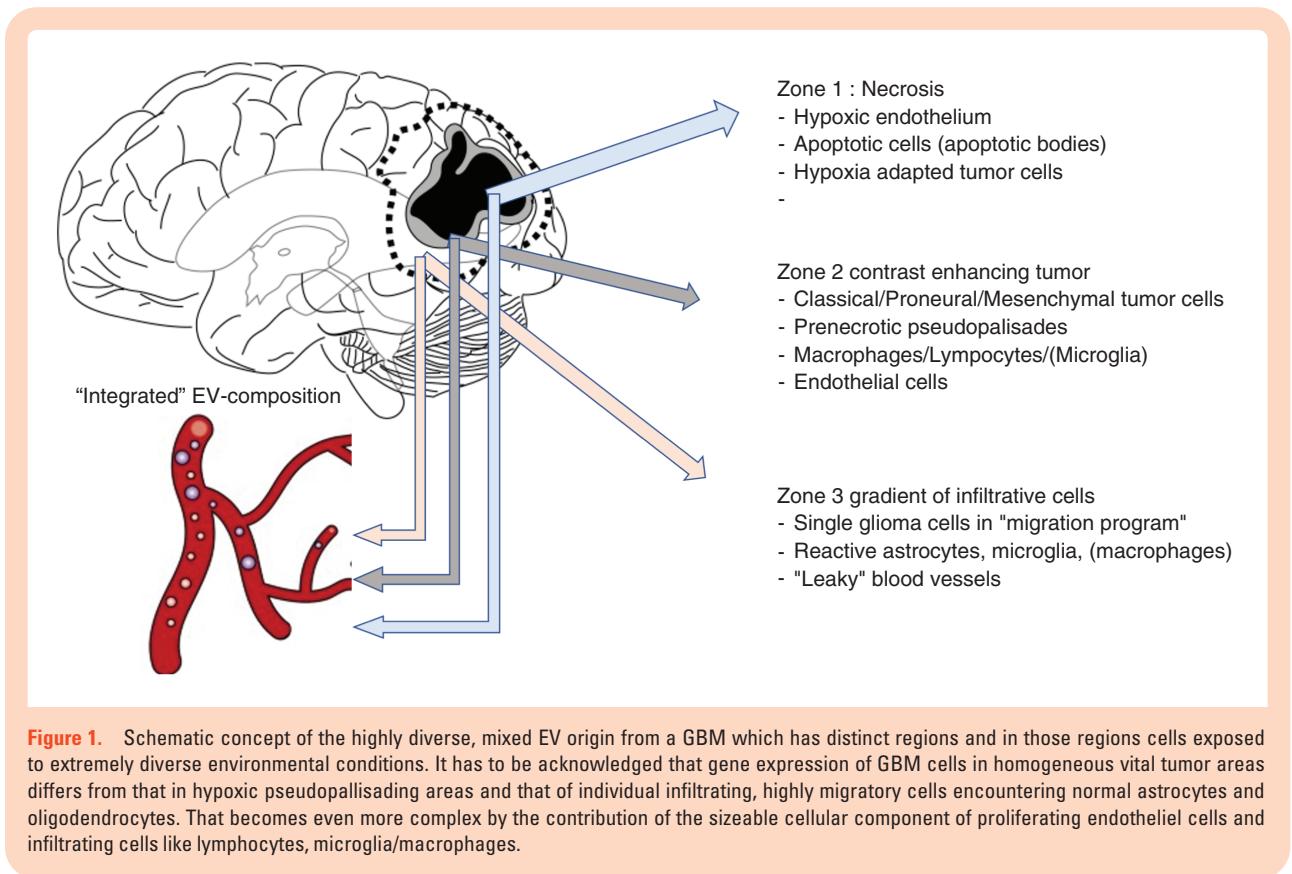
Without further expanding this topic in the context of this GBM centered review, it appears fair to state that in contrast to GBM, for breast and lung cancer, CTCs have a relevant diagnostic and monitoring potential, and furthermore with signatures predicting the likelihood to develop BMs also an implication for timely screening and continuous surveillance.

Extracellular Vesicles

In a separate development, the potential of soluble, subcellular components derived from tumors has found clinical application as diagnostic tool. Complementary to CTCs, the isolation and analysis of cell free DNA (cfDNA) carrying tumor specific mutations (ctDNA) has been technologically refined so in some entities it serves as indicative

tumor marker.³⁴ It needs to be pointed out that there are still definition issues for the term of “cell-free DNA” as it may be freely circulating with a very short half-life of only a few hours found in experimental tumor or injury paradigms.^{35,36} Else it may be part of the cargo contained in or stuck on the outside of EVs.^{37,38} Therefore most of the cfDNA or ctDNA data actually relate to DNA isolated in part from EVs and not surprisingly, in such DNA all the characteristic mutations found in GBM were found by some study either in blood or CSF as recently reviewed³⁹ and will not be much further discussed here in their own right. EVs are membrane bilayered vesicular particles with different routes of genesis extruded by all cells which have been laboriously classified over the past years by a dedicated scientific society (International Society for Extracellular Vesicles, ISEV) and a nomenclature has emerged which distinguishes mainly extracellular vesicles, exosomes and larger oncosomes, of which the former two are not exclusively related to the oncological situation but rather an intercellular communications tool. In most contexts, EVs are understood as exosomes derived from intracellular manufacture in microvesicular bodies via the ESCRT complex which then release them after fusion with the cell membrane^{40,41} and so called microvesicles which are directly folded off the cell surface (reviewed in⁴²). Their history and the very rapid gain in attention has recently been comprehensively reviewed.⁴³ As means of intercellular communication with microenvironmental but also distant signaling consequences, EVs are physiologically regularly shed from nearly all cells and enter the circulation. They carry fragments of DNA, micro-RNAs lncDNA, and proteins, thus being compact information packages which carry regulatory and potentially also transforming information throughout the body. In the context of metastasis it is even assumed that they are instrumental in preparing the “premetastatic niche”.⁴⁴ It is thus not surprising, that there are numerous reports on almost any known molecule to be found in cells also being “discovered” in EVs as reviewed and summarized for example in^{39,42,45–47} without being exhaustive to that issue. Also, experimentation with EVs in vitro and in vivo has shown them to be implicated in all cancer hallmarks like angiogenesis, replication, proliferation, evading the immune system.^{48–52}

So far, by sheer analysis of numbers, EVs correlate to the presence of tumor mass and show rapid dynamics by declining to almost undetectable levels within a day after tumor removal.^{53,54} Using the well established tetraspanins as markers (CD81, CD9, and CD63), this dynamic appears to be specific and reliable and thus serves the purpose of a “biomarker” in liquid biopsy, especially as the rise in EV counts correlates with tumor recurrence. Also, it was shown, that the analysis of such EV populations isolated from the corresponding cultured cells and harvested after several days in culture allowed methylation profiling with excellent matching of the profiles between EVs and the original tumor.⁵⁵ EVs isolated from patient plasma in the situation of an existing tumor will however be more complex and an integral of EV-shedding by tumor cells as well as reactive, enhanced production from the nontumoral infiltrative cellular constituents such as endothelial cells, microglia, lymphocytes, and the surrounding edematous sphere (Figure 1).



Because of their physiological occurrence which has long been known, and the apparently minor contribution of specific tumor cell derived EVs to the circulating EV pool, their selective isolation from the circulation is a challenge and the current focus of technological refinement.^{56–59} This has, however, not been accomplished yet for plasma-derived EVs, but as further specific purification of circulating tumor cell EVs from the complex baseline EV population are continuously technologically optimized standardization for clinical use is to be expected. This may be easier for EVs isolated from CSF as there the environment will have a less complex composition, but harvesting CSF in sufficient quantities is much more involved than sampling peripheral blood.⁶⁰

For the above-mentioned reasons, EV isolates from plasma will only yield a summary-effect. Thus characterizing EV subpopulations unequivocally related to oncometabolic pathways as biomarkers for therapy monitoring has to be approached stepwise. As EVs are continuously shed by glioma cells also in culture, EVs isolated from cell culture supernatants are an obvious tool to study EV-mediated effects, starting already with comparatively pure, albeit selected EV populations. Their use for in vivo experimentation is somewhat complex because either a syngeneic model is used with its limits of extrapolation to the human situation or heterologous systems when used are usually immunosuppressed,—eliminating the immune-interactive aspect. But already in an early series of experiments, an immunomodulation with a vaccination-like effect could be seen upon the injection of tumor derived EVs.⁶¹ Since, among many other reports, systemic

immunosuppression has been confirmed,⁶² PD-L1-positive EVs secreted by GBM tumor cells for example mediate immune invasion⁴⁹ or induce immunosuppressive monocytes which in turn inhibit T-cell proliferation.⁶³ The EVs being derived from microglia in this study illustrate as in many other reports, that EVs are instrumental to exchange of information with functional relevance in the tumor microenvironment, much of which is mediated by their micro-RNA cargo⁶⁴ like also the reprogramming of endothelial cells towards an angiogenic phenotype.⁶⁵ In another, specific pathway analysis it was shown that miR-124 as microglial EV derived cargo mediated modification of tumor metabolism leading to enhanced glutamate clearance⁶⁶ and alteration of growth behavior.

A very useful tool for in vivo experimentation, is the fluorescent labeling of defined EV-producer cells which, when orthotopically implanted form a labeled EV producing xenograft allow for the tracking of the tumor derived EVs in an experimental animal.⁶⁷ That circumvents the relatively short half-life of EVs as they will be continuously produced and a snapshot of EV distribution can be taken any time. Looking for particles with the fluorescent signal of the source tumor, it was found that EV staining could be found in cells isolated from the cervical lymph nodes supporting the concept of distant EV trafficking to extracranial sites (Figure 2, Ricklefs et al, manuscript in preparation).

It becomes apparent, that the reflection of tumor methylation profiles in EVs has a great biomarker potential, but this has so far only been possible in vitro because of lacking specific enrichment procedures from biofluids yet.

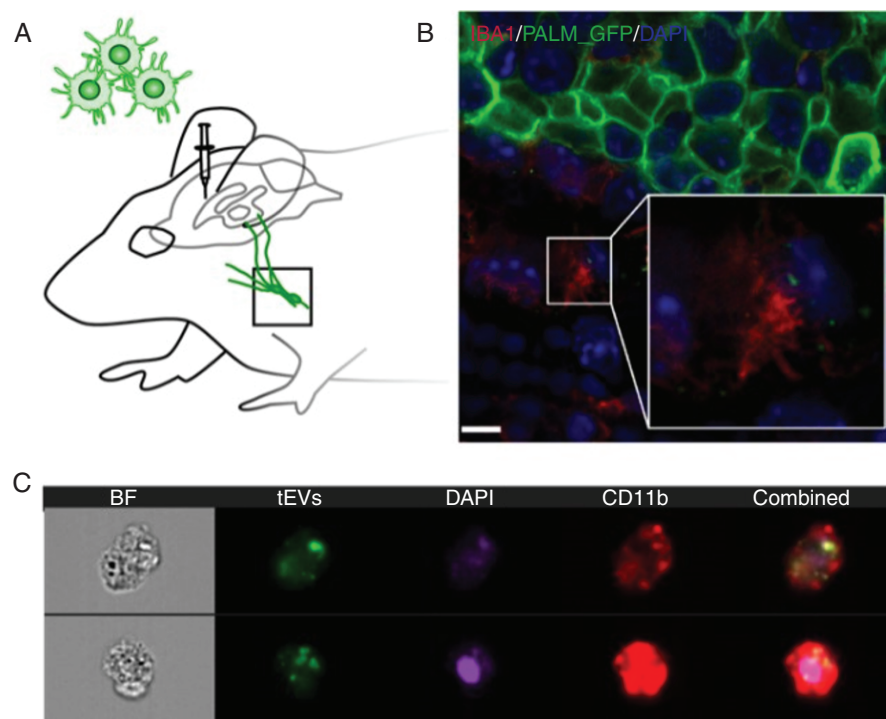


Figure 2. Fluorescently labeled EVs can be seen in cells isolated from cervical lymph nodes of tumorbearing mice. A) Palm-GFP-labeled tumor cells are stereotactically injected into mice brains. Cervical lymph nodes, as the “sentinel” lymph node of the brain are exploited for GFP EV-carrying cells. B) IBA1-positive cells (microglia/macrophages) show GFP-positive vesicular structure at the intracranial tumor border. C) CD11b-positive cells (macrophages) isolated from the cervical lymph nodes have detectable levels of GFP-positive vesicular elements seen by Imaging flow cytometry.

This as indicated is on the way to be technically resolved at some point so that beyond quantitative measures, specific qualitative biomarker information hopefully will be obtained in a standardized fashion in the future.

Discussion and Perspective

Although the therapeutic options for aggressive gliomas, in particular, GBM are limited, obtaining the maximal available information on the disease during its course is desirable. In the initial, newly diagnosed stage maximal information will always be obtained from tissue by maximal resection or biopsy. Obtaining circulating biomarkers by “liquid biopsy” at this stage allows to analyze how comprehensive the liquid biomarkers are for the whole tumor. It also allows to validate analyte isolation methods and further technological refinements as correlation with the results from tissue analysis are available. EVs isolated from the CSF in newly diagnosed cases has been shown to reliably reflect tumor biology, but at that stage, clinical use would be limited to those cases where absent mass effect and guaranteed free passage of CSF flow does not prohibit lumbar puncture. Systematic comparative longitudinal analysis of EVs from blood or CSF will solve the issue of equipoise between the respective EV sources or reveal selective/differential shedding from the complexity of different tumor compartments.

Complexity is well established and only recently, several groups looking at single cell RNAseq and multi-omics in respect to regional distribution of distinct patterns reported that “omically” defined cell types (like OPC progenitor like or NSC-like) will be regionally mixed throughout the tumor.⁶⁸ It is to be hoped that along with the “bulk flow” of EVs emerging from a tumor, the composition of the EVs,—at least from the viable parts of the tumor (Figure 1, zone 2) will give a real life mixture of the cellular states there.⁶⁹ A recent locoregional multi-omics analysis indicated spatial concordance of methylation patterns in the majority of cases but not throughout.⁷⁰ Along these lines, it is unlikely, that there will be any unique uniform marker contained in EVs that will allow to decisively alter treatment of GBM. When looking at the Heidelberg Classifier based on the 850k methylation profiling array,⁷¹ the tumors assigned to a WHO class are gathered in “clouds,” consequential to minor differences in their methylation pattern. So by inference, this, together with the given complex cellular composition of a glioblastoma with synchronous presence of molecular subtypes, that means that the contents of EVs are stochastically assembled and that there is no uniform cargo, probably not even from one individual case. The biological potential, may vary from patient to patient and even longitudinally in one patient during the disease course due to clonal selection with therapy and clonal selection through differences in advantageous opportunistic environmental adaptation of subclones.

Beyond serving as biomarkers with yet to be firmly established potential, further exploration of EV biology bears great potential also in neuro-oncology. If the modification of the tumor microenvironment towards immunological tumor tolerance and even support is mediated by EVs, that could become a novel target for tumor interference. Once it is described by which specific address EVs transform/regulate infiltrating lymphocytes, macrophages, microglia or surrounding astrocytes, biosimilar synthetic exosomes, delivered locally or systemically containing antagonistic cargo (i.e. antisense RNA to neutralize micro RNAs, antibodies, decoy receptors, blocking ligands, antisense or miRNA neutralization) could be explored for anti-tumor effects as novel therapeutics.⁷² Such novel strategies will be linked to the biomarker potential of EVs and their predictive value of microenvironmental modification. Therefore, the near goal has to be also to analyze the trafficking of the EVs and the prerequisites needed to enter the cells of the microenvironment or even further systems that convey tolerance to the tumor.

In consequence, it appears that the information obtained from liquid biopsy in gliomas will be most informative from EVs as that will represent the information that tumor cells will convey to immediate and distal environment via that intercellular communication pathway. Because of the immediacy with the relatively short half-life of only days, that is acute information useful for tumor status and response to therapy or eventually even therapeutic opportunities. In the situation of minimal disease or early stages of renewed tumor activity, a liquid biopsy from the circulation and/or from CSF may indicate evasion of current therapy, clonal selection in the developing recurrence, and hopefully in the future point to newly emerging treatment opportunities for targetable molecular alterations.

The refinement of EV characterization and technological advances to standardize isolation and enrichment over a relatively short period of not even decades is reason for optimism for a central role of this class of biomarkers also in neuro-oncology. With significantly less potential as biomarkers, CTCs isolated from GBM patients nevertheless carry important information in the context of so far unresolved clues for the prerequisites for hematogenic dissemination or in turn the potential control of their tumorigenic capacities outside the brain environment.

Acknowledgments

The Hans-Dietrich Herrmann Laboratory for Brain Tumor Research is supported by the Johannes Bauer Stiftung für Hirntumorforschung of the University of Hamburg, Germany F.L.R. and K. L. are supported by the Deutsche Forschungsgemeinschaft grant RI2616/3-1. U.K.P. received funding from the European IMI research project CANCER-ID (115749-CANCER-ID), European Union Horizon 2020 Research and Innovation program under the Marie Skłodowska-Curie grant agreement No 765492 and ERA-NET EU/TRANSCAN 2 JTC 2016 PROLIPSY. K.P. also received funding from Deutsche Krebshilfe (Nr. 70112504), Deutsche Forschungsgemeinschaft (DFG) SPP2084 µBone and ERC Advanced Investigator Grant INJURMET (No. 834974).

Conflict of interest statement. No author declares conflicts of interest.

Authorship statement. MW wrote the paper and conceptualized the research projects. KP, SR, HW and MM contributed data interpretation for the CTC aspect of the review. FLR, CM and KL contributed to the data interpretation and contributed to the EV section. All authors read and approved the final manuscript.

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