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Current understanding of circulating tumor cells – potential value in malignancies of the central nervous system

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Detection of circulating tumor cells (CTCs) in the blood via so-called “liquid biopsies” carries enormous clinical potential in malignancies of the central nervous system (CNS) because of the potential to follow disease evolution with a blood test, without the need for repeat neurosurgical procedures with their inherent risk of patient morbidity. To date, studies in non-CNS malignancies, particularly in breast cancer, show increasing reproducibility of detection methods for these rare tumor cells in the circulation. However, no method has yet received full recommendation to use in clinical practice, in part because of lack of a sufficient evidence base regarding clinical utility. In CNS malignancies, one of the main challenges is finding a suitable biomarker for identification of these cells, because automated systems, such as the widely used Cell Search system, are reliant on markers, such as the epithelial cell adhesion molecule, which are not present in CNS tumors. This review examines methods for CTC enrichment and detection, and reviews the progress in non-CNS tumors and the potential for using this technique in human brain tumors.

Keywords: circulating tumor cells, glioblastoma multiforme, glioma, liquid biopsy, epithelial-mesenchymal transition

Introduction

Detection of circulating tumor cells (CTCs) is of current great interest in central nervous system (CNS) malignancies because of recent intriguing reports, suggesting that cells from a proportion of patients with glioblastoma multiforme (GBM) may be detectable in the bloodstream (1–3). Outwit the CNS field, detection of CTCs represents a promising non-invasive technique to facilitate early diagnosis and monitoring tumor biology evolution, which is underlined by over 500 studies, registered internationally involving CTCs (4–6). The potential of the CTC approach was highlighted in an early study by Ross et al. who described peripheral blood contamination by free floating cells of

Abbreviations: BBB, blood–brain barrier; CNS, central nervous system; CTC, circulating tumor cells; ctDNA, circulating tumor DNA; DAPI, 4',6-diamidino-2-phenylindole; DTCs, disseminated tumor cells; EMT, epithelial–mesenchymal transition; EpCAM, epithelial cell adhesion molecule; FISH, fluorescent *in situ* hybridization; GBM, glioblastoma multiforme; GFAP, glial fibrillary acidic protein; hGBM, primary glioblastoma multiforme cells; IF, immunofluorescence; IHC, immunohistochemistry; ISH, *in situ* hybridization; MNC, mononuclear cells; OS, overall survival; PCR, polymerase chain reaction; PFS, progression-free survival; QPCR, quantitative PCR; RT-PCR, real-time PCR.

metastatic breast cancer in patients receiving autologous stem cell transplants (7). Due to the scarcity of CTCs in the blood the interest in this field initially shifted to enhancement of cell detection and identification using techniques, such as immunomagnetic labeling followed by polymerase chain reaction (PCR) (8, 9). RT-PCR and qPCR are widely used today as standards of CTC identification (10) although both methods are highly sensitive and suffer from a false positive rate due to the presence of contaminating cells (9).

Circulating tumor cells have now been described in most common carcinomas including breast, prostate, and colorectal carcinoma (11, 12), and most recently in CNS malignancies (13–17). There is evidence that CTC count has prognostic validity in breast cancer (18) and in particular has been related to progression-free survival (PFS) and overall survival (OS) in patients with metastatic disease (19).

At present, the only CTC detection platform to receive validation by the Food and Drug Administration of the United States of America is the CellSearch[®] system (Veridex, Raritan, NJ, USA) (20), which is a robust platform but not without its limitations, discussed further in this review. Currently, more advanced methods, e.g., CTC-chip and the EPISPOT allow isolation of still viable tumor cells enabling more detailed analysis (21, 22).

Expanding research in this field has uncovered biological dynamics of CTCs along the metastatic pathway (23) and uncovered tumor subtypes namely stem cells and disseminated tumor cells (DTCs) (24). DTCs are a CTC subpopulation found in bone marrow which may act as a dormant reservoir of malignant disease (4, 25, 26) and it is suggested that their prognostic value might be equal to CTCs (4, 24). A study by Baccelli et al. suggested a subpopulation of CTCs displaying CD44 cancer stem cell and bone homing marker, CD47, which inhibits phagocytosis and MET (a hepatocyte growth factor receptor); these have been postulated to reflect the promotion of metastatic and invasive activity (27).

Dissemination of Malignancy

Initial stages of potential metastatic tumor spread begin with a heterogeneous population of malignant cells where the dynamic changes in the tumor cell genome may give rise to metastasis induction, followed by progression and virulence (28). The development of metastatic disease has been divided into several stages, each characterized by specific genomic, epigenomic, and phenotypic alterations: persistence of proliferation-promoting signals, evasion of growth suppressors, resistance to cell death enabling replicative immortality, promotion of angiogenesis, and initiation of the invasive and metastatic process (29). Studies have demonstrated that as few as 0.01% of circulating cancer cells develop into secondary tumors with oxygenation, pH, nutrient supply, and inflammatory response constantly influencing this process (28). It is now thought that in order to enter the circulation, epithelial tumor cells may undergo epithelial–mesenchymal transition (EMT) (30). Primary epithelial malignant cells may putatively undergo transdifferentiation to a mesenchymal genotype with intermediate epithelial–mesenchymal forms present (4, 23, 25, 26, 30, 31). Furthermore, it has been postulated that to exit the circulatory system, CTCs may in fact undergo a

“reverse” mesenchymal–epithelial transition (4), suggesting that the most effective CTCs are probably of an intermediate phenotype. EMT is thought to have an origin in embryogenesis when bulk migration of developing cells occurs through compact tissue stroma and there may be upregulation of many factors including (TGF β), WNT, platelet-derived growth factor (PDGF), and interleukin-6 (IL-6) (30). While cells undergo EMT they gradually lose their epithelial markers, i.e., E-cadherin, claudin, and plakoglobin (23, 30) and acquire mesenchymal markers, such as fibronectin, cadherin 2, and serine proteinase inhibitor-1 (SERPIN 1) (32).

More recent evidence suggests that in addition to single CTCs, tumor fragments are also represented in the blood as microemboli containing stromal fibroblasts, leukocytes, and platelets (33) creating a “floating” microenvironment. These micro-fragments have been shown to evade anoikis and elimination by the immune system in the bloodstream (30, 33, 34) and promote adhesion and tissue invasion at secondary sites (30, 33, 35). Uppal et al. explored this mechanism by showing that aspirin may disrupt adherence of tumor microemboli at distant tissues (36). CTCs are relatively rare with approximately 1 CTC per 10^5 – 10^8 white blood cells (26). CTCs are phenotypically thought to be a heterogeneous population, each cell showing variable expression of biomarkers (37). However, the epithelial cell adhesion molecule (EpCAM) is a 30–40 kDa transmembrane glycoprotein commonly expressed not only on epithelioid CTCs but also on a proportion of white cells (38). It has become the target molecule for cell selection and enumeration of various detection systems primarily focused on epithelial malignancies (20, 21, 39–45).

In an optimal theoretical model, CTCs should express biomarkers not detected on other intrinsic cells in the bloodstream and not lost in the mesenchymal and circulating cell transition (46). They can be divided according to their function into prognostic, pharmacodynamic, predictive, surrogate, and monitoring biomarkers (5). The extracted cell should remain viable to allow *post hoc* molecular analysis (4, 46) and acquisition of good quality DNA rich material assures more efficient molecular identification of cells (47).

CTC Enrichment and Detection

Numerous techniques of CTC identification can be divided into broad groups according to methods of cell enrichment and cell detection, which can be used in various combinations (26, 33, 48). The most commonly shared principles of enrichment are antibody mediated or physical methods followed by secondary immunohistochemical enumeration and/or subsequent genetic analysis (33).

The CellSearch[®] platform utilizes EpCAM labeled CTC enrichment using antibody-coated magnetic beads and labeling with fluorescent-coated antibodies against cytokeratin together with 4',6-diamidino-2-phenylindole (DAPI) nuclear coating (19, 20). Although widely used, it is recognized that EpCAM-based enrichment suffers from limitations, such as relatively low sensitivity and purity, partly due to the presence of EpCAM negative tumor cells (38, 49). For example, cells expressing CD45+ EpCAM+ were

demonstrated to represent a macrophage population that may be a source of false positivity (50).

Enrichment selection methods using the anti-EpCAM antibody have been evolving through the introduction of microfluidic chips (CTC-chip, CTC-iChip®, Herringbone Chip, etc.) and of variations to known immunomagnetic and flow cytometry techniques (39, 45). Novel models have described the use of an antibody-coated intravenous wire, which is inserted directly into a vein (40). By directly exposing the probe to a constant large-volume flow of blood this method increases the probability of capturing CTC thereby addressing the issue of their very low concentration. Fisher et al. incorporated leukapheresis together with CellSearch® to address this issue (51). A variety of physical property-based enrichment methods have also been introduced, such as dielectrophoretic field flow fractionation (DEP-FFF) (52), ISET® (53), or Dean flow fractionation (54), some of which may allow cell culture of retrieved CTCs.

Figure 1 presents the most commonly described methods of CTC enrichment and detection. A standard 7.5–10 ml blood sample is processed within 2 h of withdrawal. Biological enrichment incorporates anti-epithelial, leukocytic, or mesenchymal antibodies labeled by a magnetic particle or affixed to a post or a rod (20, 39, 55). Positive enrichment relies on selective capture of CTCs, while negative enrichment through labeling of CD45 filters out cells which express leukocytic markers (22).

Modified immunomagnetic methods can achieve high degree of CTC purification and to allow downstream analysis, while some

techniques preclude cell culture of retrieved CTCs. Combination of techniques, such as MoFloXDP cell sorting, with qPCR allows high-throughput analysis through single cell-array based comparative genomic hybridization (56, 57). In a related method, the AdnaTest utilizes double EMA and EpCAM magnetic bead enrichment followed by RT-PCR multigene panel (56). Microfluidic on-chip methods have been developed offering a single device solution and efficient analysis pairing immunomagnetic enrichment with IHC or PCR (21, 41–43, 58). Certain chips offer single cell high-throughput analysis through physical enrichment taking advantage of cell size and deformity, again with biological properties preserved (59). Others combine filter-based methods with on-filter immunofluorescence (60). The micro-Hall detector (μ HD) chip enriches CTCs with immunomagnetic nanoparticles allowing up to 10^7 cell/min analysis preserving antigenicity permitting the use of bespoke immunoprofiles (61). An additional advantage of this approach is the ability to analyze unpurified samples which reduces processing time (61). A different nanoparticle method uses gold particles with single strand DNA, which bind to intracellular mRNA in live cells. The entry of nanoparticles into cells does not induce cell death preserving the isolated CTCs for downstream phenotyping (62).

Apart from CTCs, circulating tumor DNA (ctDNA) fragments offer an attractive quantification tool through digital PCR assay and targeted deep sequencing (63), which is based on the samples obtained from 30 patients with metastatic breast cancer. Dawson et al. found ctDNA to be of superior prognostic value

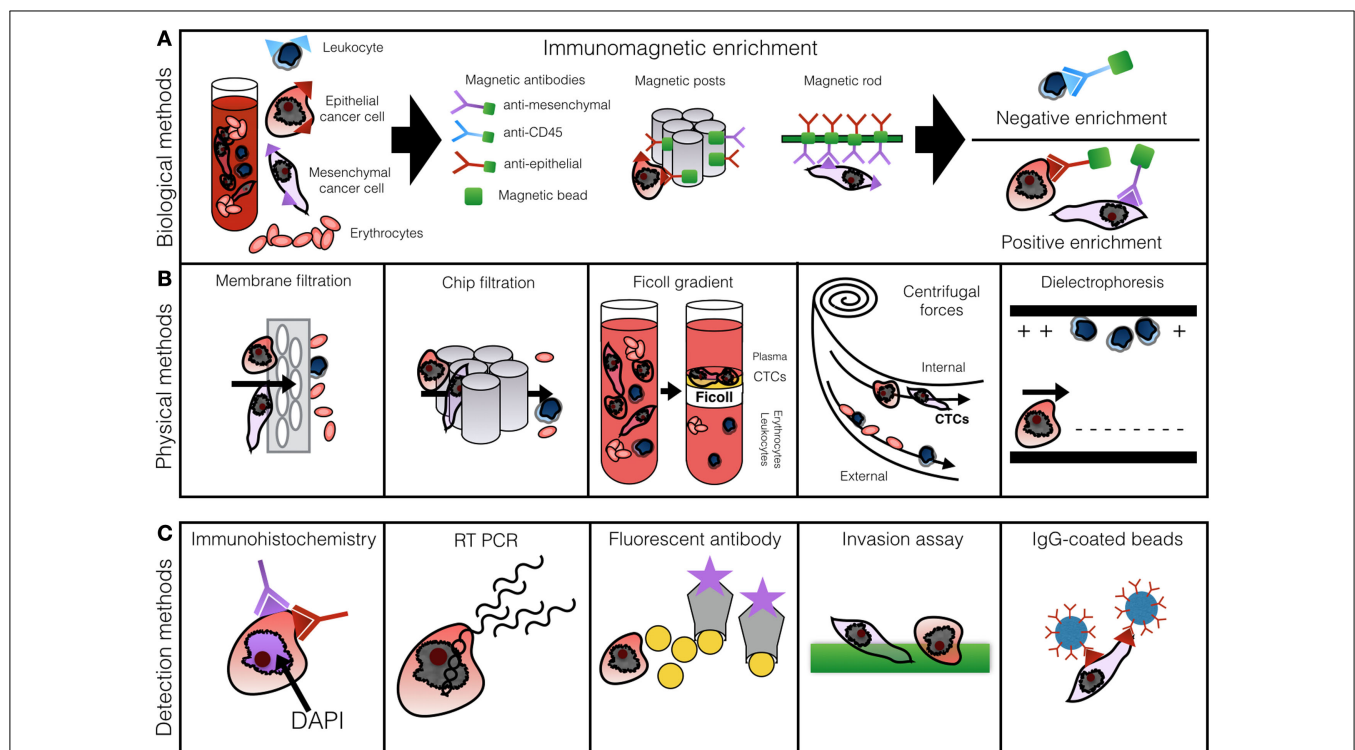


FIGURE 1 | Row (A) demonstrates methods of biological CTC enrichment using magnetically labeled antibodies captured in a magnetic chamber or by posts or rods. Row (B) illustrates physical enrichment methods: membrane filtration, microfluidics, Ficoll gradient

centrifugation, Dean drag forces separation, and dielectrophoresis. Row (C) outlines the most common principles of cell enumeration: IHC, RT-PCR, fluorescent antibody labeling, invasion assay, or antibody-coated beads. Based on Alix-Panabieres and Pantel (46) with permission.

to Ca15.3 and CTCs (63). However, the biological properties of CTCs have the potential of specific tumor phenotyping (63). Investigation into other uses, such as diagnosis of malignancies of unknown primaries, offers another interesting potential use of this methodology (64).

CTCs as Predictors of Survival in Non-CNS Malignancies

In 2007, the American Society of Clinical Oncology issued treatment guidelines, which did not recommend routine detection of CTCs in breast cancer patients in part due to a lack of evidence base as to their prognostic utility (65). However, numerous publications including meta-analyses have emerged since which may be addressed in updated guidelines. Following a recent international multi center study, there is now substantial evidence that detection of five or more CTCs in the blood of breast cancer patients leads to decreased OS and PFS at different stages of follow-up (66). A recent meta-analysis by Zhang et al. found that CellSearch® enrichment combined with RT-PCR was superior in predicting PFS, while prediction of OS was similar regardless of the methods used (67). Prediction of OS was most significant when CTCs were measured at cancer baseline compared with other stages of disease (67). CTCs have been found in patients in all clinical stages including patients with T1 and T2 operable disease regardless of tumor stage, grade, lymph node, or receptor status (68). Some authors suggested a single detected CTC carries higher disease progression risk in non-metastatic chemosensitive (69) and locally advanced (70) breast cancer. Furthermore, in a related study by Pierga et al. showed predictive significance even if detected at a rate of ≥ 1 CTCs (71).

Presently, ongoing trials are focusing on particular stages of treatment and analyze methods of CTC detection in metastatic breast cancer (CTC-EMT, CTC-CEC-AND), provide input on clinical aspect relating to cost-effectiveness (STIC CTC), and evaluate patients at specific clinical points providing information on prognostication and treatment guidance (Detect III, CirCe01, Treat CTC, COMET1 P2) (25). In aggressive triple negative cancers, baseline and early follow-up measurement of CTCs identifies groups of patients with higher tumor chemoresistance (TBCRC 019) (72).

The LANDSCAPE trial investigated patients with metastatic disease to the brain comparing CTC levels before treatment and after lapatinib and capecitabine at 3 weeks in Her2 positive tumors (71). The results were compared with levels of soluble serum biomarkers. The authors found CTCs predict treatment response more accurately avoiding post treatment biomarker spike (71). Interestingly, CTCs occurred less frequently in patients with isolated brain metastases presumably due to properties of the blood-brain barrier (71, 73). Metastatic breast cancer cells variably express EpCAM but may have a more distinct phenotype HER2+/EGFR+/HPSE+/Notch1+ (74). An additional, potentially useful marker, aldehyde dehydrogenase 1 (ALDH1), is of interest as cells lacking this enzyme are unable to form tumors (75).

Second to breast, CTCs have been commonly utilized in prostate carcinoma with the same CTC cut-off of five or more

CTCs found to be correlating significantly with prognosis (76). The predictive value of CTCs was described in castration resistant prostate cancer following prostate-specific antigen (PSA) and lactate dehydrogenase (LDH) serum levels (77). Although the CellSearch® system appears to be the method of choice in prostate cancer, more specific markers may be necessary, such as cadherin-11, which is expressed not only on prostate cells and osteoblasts but also on prostate cancer cells exhibiting EMT (78).

Colorectal cancer CTCs traverse the portal circulation with a proportion of the cells being filtered by the liver (79). In colorectal cancer, a value of 3 CTCs or above has commonly been used (80). A large study from the USA showed an independent prognostic effect of CTC counts regardless of serum carcinoembryonic antigen (CEA) levels (81). However, measurement of CTCs prior to resection of liver metastases does not appear to show prognostic effect (82). Colorectal CTCs have been demonstrated to show similar KRAS and BRAF gene status to the primary tumor with 68–100% concordance (5, 83). This may allow identification of patients more likely to be resistant to EGFR inhibitors, but methods of more effective enrichment are required to prevent false negative mutation results (83).

Several studies emerged which confirmed the presence of CTCs in both small cell and non-small cell lung carcinomas. Hou et al. found 85% of patients with confirmed cancer had detectable circulating cells at baseline (84). These authors also devised a bespoke method of establishing the best predictive CTC cut-off, arguing that the values should vary according to individual biological properties of cancers (84). Lung cancer CTCs were also shown to be suitable for EGFR receptor status analysis (85).

To date, CTCs have also been confirmed in ovarian, esophageal, urothelial, pancreatic, head and neck (13–17) carcinomas using a mixture of CellSearch® platform paired with PCR or microfluidic technologies. CTC thresholds range from 1 to 5 CTCs as cut-off but studies are conducted on small groups and require validation.

Potential Value of CTCs in CNS

Despite the fact that systemic metastases are rare in GBM, a few recent studies have successfully isolated CTCs from peripheral blood of both primary and recurrent adult GBM and diffuse glioma, which could yield great potential for disease monitoring to guide treatment (see **Table 1**). A key issue is finding an appropriate CNS biomarker to identify the CTCs, because CNS malignancies do not express EpCAM, unlike many epithelial malignancies, which commonly metastasize (3, 86). In a large study by Müller et al., CTCs were identified in 29/141 (20.6%) of GBM patients using physical separation methods (Ficoll gradient) followed by immunostaining for glial fibrillary acidic protein (GFAP) (3). In this case, the use of GFAP for CTC identification was supported by its absence in control participants, and the presence of EGFR amplifications in the tumor cells isolated using GFAP (3). The mobilization of CTCs into the peripheral blood, which still maintains EGFR amplifications supports the hypothesis that they do maintain growth potential (3).

Moreover, authors from Massachusetts Institute of Technology recently published a set of biomarkers found on CTCs with the use of a CTC-iChip® (87). The STEAM panel consisted of sex

TABLE 1 | Summary of studies attempting to isolate CTCs from patients with high-grade gliomas.

Author	Cell enrichment	Cell characterization	Results	Limitations
PUBLICATIONS POSITIVELY IDENTIFYING CTCs IN BRAIN TUMOR PATIENTS				
Müller et al. (3)	MNCs isolated by Ficoll density gradient centrifugation Cytospins prepared from MNCs GFAP positive single cells isolated by micromanipulation	GBM patients Chromogenic and fluorescent IHC (GFAP, CD45, EGFR) Further characterization of CTCs and associated tumor Comparative genomic hybridization Sequence analysis FISH	$n = 29/141$ Observed association between EGFR amplification and release of CTCs Common genomic aberrations in CTCs and GBM tumors	Low detection rate
Sullivan et al. (87)	Blood processed through a CTC-iChip® (magnetically tagged CD45 and CD16) IF-guided single-cell micromanipulation used to isolate single CTCs (EGFR, MET and CDH11)	GBM patients IHC glioma marker panel [SOX2, Tubulin, beta-3, EGFR, A2B5 and c-MET (STEAM)] FISH used to determine EGFR gene amplification in CTCs from known amplified cases Further CTC characterization by qRT-PCR and dual color RNA-ISH assay	$n = 26/87$ RNA-ISH demonstrated an enrichment for mesenchymal transcripts and a reduction of neural differentiation markers	Limited dataset Could not determine whether surgical or radiation induced disruption of the blood–brain barrier enhances CTC dissemination
MacArthur et al. (88)	Blood samples centrifuged in OncoQuick tubes	High-grade glioma patients Incubated with a telomerase-responsive adenoviral probe (via GFP expression) Secondary Immunofluorescence (Nestin and EGFR)	$n = 8/11$ pre-radiotherapy $n = 1/8$ post-radiotherapy EGFR amplification in CTCs correlates with solid tumors	Limited pilot data Need more serial measurements throughout the treatment and disease course for each patient Telomerase is elevated in other tumor histologies
PUBLICATIONS NEGATIVELY IDENTIFYING CTCs IN BRAIN TUMOR PATIENTS				
Böhm et al. (89)	Total cellular RNA extracted from whole blood using the QIAmp RNA blood mini kit (Qiagen)	High-grade astrocytoma and GBM patients RT-qPCR assay for the detection of mRNA encoding GFAP and B2M (positive control)	$n = 0/20$	Sample size Insufficient technology
Martens et al. (90)	Cytocentrifugation	Astrocytoma patient Chromogenic and fluorescent IHC (GFAP)	$n = 0/1$	Only one patient and sample tested Insufficient technology

determining region Y-box 2 (SOX2), tubulin beta-3, EGFR, A2B5, and c-Met and found specifically on high-grade glioma cells (87). Circulating glioma tumor cells were found to harbor elevated SERPINE1, TGFBI, TGFBR2, and vimentin, which are associated with an aggressive mesenchymal phenotype (87). The authors suggest that there may be a subset of mesenchymal cells present in disseminated GBM that have the ability to invade the vascular system and proliferate outside the brain as systemic lesions (87).

An interesting approach used in the pilot study by MacArthur et al. identified CTCs with an adenoviral telomerase-responsive probe that consisted of the expression cassette for green fluorescent protein (GFP) as well as the hTERT promoter driving expression of E1A and E1B for viral replication and amplification of the GFP signal that can detect the increased telomerase activity in the CTCs following physical separation with OncoQuick tubes (88). This was combined with immunofluorescence for GFAP and nestin which helped to delineate the glial origin of the CTCs (88). The MacArthur study identified circulating glioma cells in 8 of 11 (72%) pre-radiotherapy high-grade glioma patients, compared

with 1 of 8 (12%) in the post-radiotherapy cohort, demonstrating the ability of the liquid biopsy to identify patients at risk of recurrence/with high tumor burdens (88).

There is in addition a potential of the use of CTCs in the identification of patients with a phenomenon known as pseudo-progression – when the radiological features mimic tumor recurrence – but, in fact, the tumor may be undergoing a non-malignant inflammatory change (2).

Cerebrospinal fluid (CSF) is also a potential source for glioma CTCs biomarkers; however, this has not yet been evaluated in the literature to date (2, 91, 92).

Conclusion

Detection of CTCs via so-called “liquid biopsies” carries enormous clinical potential in CNS malignancies and requires urgent further research. To date, studies in non-CNS malignancies, particularly in breast cancer, show increasing reproducibility of detection methods for these rare tumor cells in the circulation.

However, no method has yet received full recommendation to use in clinical practice, in part because of lack of a sufficient evidence base regarding clinical utility.

In CNS malignancies, one of the main challenges is finding a suitable biomarker for identification of these cells, because automated systems, such as the widely used Cell Search system, are reliant on markers, such as EpCAM, which are not present in CNS tumors. There are ongoing promising initial studies which have identified CTCs in the peripheral blood of glioma patients using physical separation techniques followed by IF for markers, such as GFAP, nestin, and a telomerase promoter-based assay, or iCHIP

using the STEAM panel that consisted of SOX2, tubulin beta-3, EGFR, A2B5, and c-Met.

Author Contributions

LA: manuscript research, writing, revision, and figure design; HW: table, manuscript review, and revision; AF: manuscript review and revision; HE: manuscript review and revision; HH: manuscript review and revision; CP: manuscript review and revision; JH: manuscript review and revision; KK: manuscript writing, review, and revision.

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