Construction and Clinical Verification of Cytoplasm Protein GFAP as Circulating Tumor Cell Separation target for Pediatric Neuroepithelial Tumors

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Abstract

BACKGROUND: Pediatric Neuroepithelial Tumors (NT) are one of the most prevalent diseases among children. Developing a highly efficient cerebrospinal fluid (CSF) detection system with diagnosis and prediction function is very important. Circulating tumor cell (CTC) in CSF is a good choice. In contrast to the past use of epithelial EpCAM as CTC separation target, an cytoplasm protein of GFAP antibody was first selected to construct highly-sensitive immunomagnetic liposomes (IMLs). The validation and efficiency of this system in capturing CTCs for NT were measured both in vitro and in vivo. The associations between the numbers of CTCs in patients with their clinical characteristics were further analyzed.

RESULTS: Our data show that CTCs can be successfully isolated from CSF and blood samples from 29 children with NT. The numbers of CTCs in CSF were significantly higher than those in blood. The level of CTCs in CSF was related to the type and location of the tumor rather than its stage. Genetic testing in GFAP CTC-DNA by sanger sequencing, q-PCR and NGS methods indicated that the isolated CTCs (GFAP+/EGFR+) are the related tumor cell. For example, the high expression of NPR3 gene in CSF CTC was consistant with tumor tissue.

CONCLUSIONS: GFAP-IML isolation of CTCs, combined with an EGFR immunofluorescence assay of antitumor marker, can serve as a brand-new method for the identification of CTCs for brain tumors. Via lumbar puncture, a minimally invasive procedure, this technique can be clinically significant in diagnosis and efficacy assessments of pediatric NT.

Background

Liquid biopsy is considered as a promising technique to decipher the characteristics of malignant tumors [1]. Apart from blood testing, it can be extended to monitor other body fluids, such as cerebrospinal fluid (CSF) for tumors in the central nervous system,[2] including astrocytoma, ependymoma, medulloblastoma and others [3]. For these brain tumors in children, [4, 5] diverse clinical manifestations with poor subjective and objective descriptions of symptoms often make the diagnosis difficult and lead to misdiagnosis [6]. Therefore, the clinical assessment of pediatric brain tumors mainly relies on radiological examinations, such as CT and MRI. Nevertheless, factors such as radiation necrosis, hemorrhage, and inflammation may vastly affect radiologists, preventing a correct interpretation [7]. In addition, the lack of circulating serum markers limits valuable methods for early clinical assessment and reduces the available options for monitoring disease progression.

Circulating tumor cells (CTCs) refer to tumor cells that are found in the peripheral circulation or other body fluids and are derived or detached from primary tumor sites [8]. As a promising liquid biopsy technique, detecting and analyzing CTCs is an ideal way of identifying the nature of tumors with a minimally invasive approach [9]. In the past five years, CTC detection has been widely used to diagnose and continually assess tumor progression in various malignant tumors, such as breast cancer,[10] lung

cancer,[11] liver cancer,[12] prostate carcinoma[13] and, recently, brain tumors [14]. The conventional CTC positive sorting method mainly involves a positive enrichment method designed on the basis of high expression markers on the surface of tumor cells such as EpCAM; that is, EpCAM antibodies or short peptides are labeled on the surface of the substrate, such as immunomagnetic spheres, or microfluidic chips [15, 16]. However, the positive sorting method based on CTC surface markers (EpCAM, EGFR and other surface markers) cannot obtain more representative tumor cells, resulting in missed selection of many cells with different molecular phenotypes, which has severely restricted the CTC application value [17, 18]. Therefore, cytoplasmic markers have become suitable candidates for CTC separation, especially in tumors with unreliable surface markers. In this study, for the first time, we introduced the endocytic GFAP antibody of neuroepithelial tumors as a positive CTC sorting marker to verify its feasibility in the diagnosis of brain tumors in children.

Due to the existence of a blood-brain barrier in the central nervous system, [19] detection of CTCs in CSF was thought to be much more sufficient than detection in peripheral blood [20]. Recent studies on brain metastasis of epithelial tumors such as breast and lung cancer have demonstrated the successful isolation and identification of CTCs from CSF of patients [21, 22]. However, the detection and application of CTCs in CSF were unclear in primary tumors of the central nervous system, especially in children. Therefore, further investigation of CSF CTCs from pediatric brain tumors would be of great importance in the clinical management of patients. Herein, the cytoplasmic protein of GFAP was selected to construct an immunomagnetic liposome, which was used as a CTC isolation system for pediatric neuroepithelial tumors (NT).

Materials And Methods

PREPARATION OF IMMUNOMAGNETIC LIPOSOMES (IML)

GFAP-IMLs were prepared by using the reverse-phase evaporation (REV) method. Briefly, 5 mg dioleoylphosphocholine (DOPC) (Avanti Company) and 5 mg cholesterol were obtained and added into a 50 ml 3-neck flask. After removing ethanol, 1.0 mg Fe_3O_4 -hydrophobic magnetic nanoparticles (HMNs) were dissolved in 3.0 ml CH_2Cl_2 and transferred into the previously prepared 3-neck flask. The mixture in the flask was sonicated on ice. Simultaneously, 2 mg GFAP antibody (Abcam, ab7260) modified glycidyl hexadecyl dimethylammonium chloride (GHDC) was dissolved in 6 ml ddH₂O and gradually added to this flask. After emulsification, rotary evaporation was used to remove the residual CH_2Cl_2 from the emulsion. The solution was magnetically separated and washed three times, and GFAP-IMLs were obtained. Similarly, EpCAM (Abcam, ab71916) modified IMLs were constructed. The detailed preparation process and reagent consumption are described in a previous study [23].

EVALUATION OF THE INTERACTION BETWEEN IMLS AND BRAIN TUMOR CELLS

Commercial glioma cells U87 and U251 were routinely cultured in DMEM complete culture medium supplemented with 10% FBS and 1% penicillin-streptomycin in a humidified 5% CO₂ incubator at 37 °C. The cytotoxicity assay of IMLs is described in the supplementary file. Sterile slides were placed in a 24-well plate, and 1×10^4 U87 cells were seeded into each well containing 1 ml culture medium. These cells were cultured in a 5% CO₂ incubator at 37 °C for 24 hrs. After refreshment with new medium, IMLs were added to each well at 20 μ l/well, and an equal volume of PBS was added to the control wells. After 48 hrs of incubation, the medium in each well was discarded and washed with PBS. After fixed the cell with paraformaldehyde, 100 μ l DAPI staining solution was added to each well and incubated for 5 minutes. After discarding the staining solution and washed with PBS, cell slides were removed from the wells and placed in an inverted position on a slide-proof glass slide coated with antifade mountant. These slides were observed by a confocal laser scanning microscope.

Rhodamine 123 (R123) (Sigma) was encapsulated in the GFAP-IMLs. After dialysis for 5 hrs to remove the excess free R123, R123-labeled GFAP-IMLs were mixed with U87-GFP cells and incubated at 37 °C for different minutes. The final dyeing effect of U87-GFP cells was observed under a fluorescence microscope after washing with PBS.

EXPERIMENT ON THE RECOVERY RATE OF SIMULATED CTCs BY IML

Different (10–200) suspended U87 cells were mixed with 7.5 ml PBS with anticoagulant to mimic the CTCs in CSF and blood. The abilities of GFAP-IMLs, EpCAM-IMLs, EGFR-IMLs and IMLs to capture CTCs were measured. The cell suspension was dropped onto a slide-proof polylysine-coated glass slide, which was subjected to a fluorescence microscope after drying out. The experimental procedure of CTC detection is same in our previous study [23] and described in Supplementary file.

U87-GFP CELL SEPARATION EFFICIENCY OF GFAP-IML IN VITRO AND IN VIVO

U87-GFP cells at the logarithmic growth stage were digested with trypsin to make the cell suspension. A total of 0.2 ml of the prepared U87-GFP cell suspension (1×10^7 /ml) was subcutaneously injected into the right back skin of 4-week-old female BALB/c nude mice (Supplied by SLRC Laboratory Animal). Tumor volume was calculated according to the following formula: Tumor volume $V(\text{mm}^3) = \pi/6 \times \text{Length (mm)} \times \text{Width (mm)}^2$. Measurement was performed once every 2 or 3 days, and the growth rate was calculated according to the following formula: Growth rate(%) = Mean tumor volume(mm^3) / Survival time of tumor-Bearing mice (days).

Blood samples (0.1 ml) were collected from nude mice eyeball, from which CTCs were isolated and identified by IML incubation followed by magnetic separation. Furthermore, 30 μ l DAPI, 10 μ l EGFR-FITC (Abcam, ab11400) solution, and 10 μ l CD45-PE were used to stain isolated cells for 15 minutes away

from light. After washing three times with PBS, the cell suspension was dropped onto a slide-proof glass slide and observed under a fluorescence microscope.

Blood samples collected from nude mice were centrifuged at 1000 rpm for 10 minutes. The supernatant was transferred carefully into a 1.5 ml centrifuge tube, and an equal volume of PBS was added and mixed. Then, 30 μ l IMLs was added and incubated at RT for 25 minutes and mixed once every 5 minutes. The rest of the experiment is the same as above of cell recovery section.

DETECTION OF CTCs IN TUMOR PATIENTS

This study was approved by the Ethics Committee of Xinhua Hospital Affiliated to Shanghai Jiaotong University School of Medicine, and all patients involved in this study signed informed consent. For each patient before tumor resection, 7.5 ml of CSF through lumbar puncture and peripheral blood were collected in an EDTA anticoagulant tube for immediate laboratory examination. Self-prepared anti-GFAP, EpCAM and EGFR IMLs were used to enrich and screen the CTCs in CSF and peripheral blood samples. DAPI was used to identify the intact cells with nuclei, and then cells were stained with fluorescently labeled anti-CD45 and EGFR-FITC (for GFAP-IML)/GFAP-FITC (for EpCAM/EGFR-IML separation) monoclonal antibodies to distinguish the neuroepithelial tumor cells from the white blood cells. CTCs that met the evaluation criteria, i.e., GFAP+, EGFR+, DAPI + and CD45-, were counted by using a complementary polychromatic fluorescence cell counting instrument.

DNA EXTRACTION AND GENE DETECTION IN CTCs

The CTCs enriched by GFAP-IMLs were used to extract DNA, and the DNA extraction methods referred to the instructions of the DNA extraction kit (EZ Bioscience, No. B0007). PCR-related amplification reagents were purchased from Yeasen Biotech (Shanghai) Co., Ltd. PCR primers were purchased from Sangon Biotech (Shanghai) Co., Ltd. H3F3A, HIST1H3B and HIST1H3C, as well as NPR3 primers are described in previous literature[24]. The primer sequence can be seen in the supplementary file.

STATISTICAL ANALYSIS

Data from each group were processed by SPSS 19.0 statistical software and presented by Prism 7.0 software. Measurement data are presented as the mean $\pm$ SD and were compared by the rank sum test. A p value < 0.05 was considered statistically significant. A heatmap was produced by R 3.3, and the number of CTCs was transferred by log transformation. For other methods employed in this manuscript, please refer to the supplementary information.

Results

CONSTRUCTION AND EVALUATION OF GFAP-IMLS

The preparation process of IMLs is shown in Figure S1. This study was based on GFAP antibody immunolipid magnetic liposomes to construct children's brain tumor CTC separation and identification

system. The microstructure and physicochemical characterization of GFAP-IMLs are shown in Fig. 1. Clear protein bands were observed in GFAP-GHDC and GFAP-IMLs in protein electrophoresis photo, indicating that the GFAP antibody was successfully inserted into the lipid bilayer of IMLs (Fig. 1A). The UV absorption spectrum of IMLs showed that the GFAP antibody, GFAP-GHDC and GFAP-IMLs had clear UV absorption peaks near 280 nm. However, with the modification of antibodies and the influence of nanospheres on UV absorption, the absorption peaks of the antibody derivatives and IMLs at 280 nm become weaker, wider and slightly shifted (Fig. 1B). The molecular configurations of GFAP antibody, GFAP-GHDC and GFAP-IMLs were studied by in situ electrochemical Raman spectroscopy. Raman spectra revealed the same characteristic peaks for all samples (Fig. 1C). The magnetic saturation curve showed that the prepared GFAP-IMLs had a high saturation magnetization, and no hysteresis was detected in the curves of Fe_3O_4 raw magnetic beads and GFAP-IMLs (Fig. 1D). The hysteresis curve was closed. The residual magnetic force and coercive force were zero within the allowed range of the instrument, suggesting good superparamagnetic properties. The maximum specific saturation magnetization of magnetofluid Fe_3O_4 was 51.3 emu/g and that of GFAP-IMLs was 30.9 emu/g, accounting for only 60.2% of the pure magnetofluid. The EpCAM-IMLs and EGFR-IMLs showed similar magnetic properties.

AFM revealed similar spherical shapes for these three IMLs, with relatively low distribution uniformity sizes (Fig. 1E-G). The particles were larger than 100 nm in diameter, and the surface of the beads was coarse. When the particle image was further enlarged (lower right corner), it was revealed that the shapes of these IMLs were irregular, and the magnetic bead surfaces had an antibody lipid membrane. The particle size and zeta potential of the three IMLs were not significantly different, as presented in the respective AFM images. A smaller particle size was more beneficial to the interaction between the beads and cells to improve the separation efficiency of CTCs. The cytotoxicity of the IMLs was shown in Figure S2.

The fluorescence intensity of U87 cells increased gradually over time, indicating an increase in the internalization of GFAP-IMLs. The bright-field view demonstrated the reduction of GFAP-IMLs outside the cells. A large number of IMLs were adhered on the surface of the cells at 10 minutes. The penetration of IMLs into the cells was significantly increased after 20 minutes. Therefore, to separate the target cells, the incubation time of GFAP-IMLs could be limited to 30 minutes. Magnetic separation with a higher cell recovery rate was conducted after 10 minutes of incubation (Fig. 2).

Spontaneous signals of GFP in U87-GFP cells were observed from 0 to 30 minutes through immunofluorescence. At 5 minutes of incubation time, R123 could not be observed. However, with the extension of incubation time, the red fluorescence became visible and gradually enhanced. At 15 minutes, the R123 fluorescence was not strong enough, but the cell contour of U87 could be seen, indicating that the GFAP-IMLs began to enter the interior of the U87 cells. The entire cell contour was clearly visible at 25–30 minutes, suggesting that the GFAP-IMLs had accumulated inside the U87 cells (Figure S3).

The results showed that three IMLs were able to capture U87 cells suspended in PBS under different concentration gradients. At the same antibody content on IMLs with the same magnetic quality, the

average efficiency of GFAP-IMLs, EpCAM-IMLs, EGFR-IMLs and IMLs in capturing U87 was 87.9%, 63.8%, 49.4% and 30.8%, respectively. The capture efficiency of GFAP-IMLs in PBS was higher than that of EGFR-IMLs and EpCAM-IMLs (Fig. 3A).

U87 cells were subcutaneously injected into 4-week-old nude mice. Three kinds of IMLs were used to mimic the capture of CTCs in the blood from nude mice after 3, 15, 30 and 40 days of subcutaneous injection. All IMLs could capture CTCs in the blood, and the number of CTCs captured by GFAP-IMLs was significantly higher than those captured by EGFR-IMLs and EpCAM-IMLs (Fig. 3B). Fluorescence images also showed that CTCs captured by GFAP-IMLs from the blood and tumor tissues of nude mice were similar in cell morphology to U87-GFP cells with spontaneous green fluorescence (Fig. 3C). The numbers of CTCs captured were positively correlated with the size of inoculated tumors, which were 0 mm³, 50 mm³, 100 mm³, and 200 mm³ in nude mice. In addition, when the tumor volume exceeded 50 mm³, the numbers of CTCs isolated by GFAP-IMLs were significantly higher than those isolated by the other two IMLs ($p < 0.05$). Through in vivo imaging of nude mice, it was confirmed that the tumor was composed of U87 cells with autofluorescence (Fig. 3D).

PRELIMINARY CLINICAL APPLICATION OF IMLS IN NT

Twenty-nine children (3 abnormal data points, which were much higher than the average, were deleted) with neuroepithelial tumors were recruited in this study (Table S1). GFAP-IMLs were applied to capture CTCs in both peripheral blood and CSF from these patients. EpCAM and EGFR IMLs were used as the control group. As a verification approach, captured CTCs were labeled by anti-EGFR antibody conjugated FITC and anti-CD45 antibody conjugated by PE. Representative staining from certain patients showed successful detection of CTCs, but not leukocytes, through the isolation process (Fig. 4).

The numbers of captured cells in peripheral blood and CSF from each sample, together with those in the control group, were calculated and are summarized in the scattergram and heat map. The number of CTCs from embryonal tumors was lower overall than any other type of tumor (Fig. 5A). For other neuroepithelial tumors, it was interesting to find that in pilocytic astrocytoma (PA) cases with KIAA1549-BRAF fusions, more CTCs were recruited than those in wild-type cases (Fig. 5B). Additionally, more CTCs could be captured in CSF samples than in peripheral blood samples from PA patients (Fig. 5C). These results indicated that the CTC separation system might be a minimally invasive procedure for diagnosing children with images revealing suspected PA cases. Furthermore, this system may provide a novel method for posttreatment assessment of efficacy. Moreover, in medulloblastoma, group 4 cases tended to have more CTCs than non-group 4 cases in both CSF and peripheral blood samples (Fig. 5D & E). This finding indicates that group 4 medulloblastoma has a higher frequency of metastatic disease at diagnosis. In addition, one patient exhibiting cerebellar glioblastoma with CSF dissemination also showed a higher CTC number (Figure S4 A & B).

As shown in Fig. 6A & B, there was no significant difference in the CTC cells sorted by different immunomagnetic spheres in the blood. In CSF, the number of CTCs sorted by the GFAP immunomagnetic sphere was significantly higher than that of the EpCAM and EGFR magnetic spheres ($P < 0.01$). To our

surprise, the number of CTCs sorted by GFAP magnetic spheres was not significantly associated with tumor stage, either in cerebrospinal fluid or in blood (Fig. 6C). The number of CTCs sorted by GFAP magnetic spheres was related to the age of the children (Fig. 6D). Children less than 3 year-old had more CTCs in their CSF than did those in other age groups ($P < 0.01$).

To strengthen evidences in supporting the origination of isolated cells, distinct genetic alterations were tested in CTCs according to the genetic characteristics in original tumors. We found that the H3F3A gene showed K27M mutations in CTCs (captured from CSF) and in tumor tissue samples from DIPG patients (Figure S5). What's more, CTCs from a patient diagnosed with Medulloblastoma were revealed with over-expressed NPR3 by RT-PCR, which was consistent to immunohistochemistry staining of NPR3 protein in tumor tissue (Fig. 6E). What's more, two same gene mutation of KMT2A and TPRSS2 were checked out in GFAP separated CTC and tumor tissue. CIC mutation was found in CTC, but not in tumor tissue. (Table S1)

Discussion

In this study, GFAP antibody combined with the lipid material GHDC and DOPC with low cytotoxicity and high biocompatibility was selected as the matrix of magnetic microspheres to construct GFAP-IMLs for CTC separation. It is stable, the surface antibody content is controllable, and it has the crystallization properties of magnetic particles. It can react with children's brain tumor cells to form antigen-antibody specific binding, and has high capture efficiency. A high-efficiency and accurate CTC detection system for children's brain tumors was formed.

These in vitro and in vivo tests indicated that the GFAP-IMLs were more specific in capturing U87 cells. In summary, through blood CTC capture, we confirmed that CTCs could be released into the peripheral blood during the process of tumor development and volume enlargement. Thus, these three IMLs constructed in the present study could efficiently isolate CTCs in both in vitro and in vivo contexts, which demonstrated the necessity of clinical tests.

The preliminary clinical application of GFAP-IMLs for CTC separation was verified by twenty-nine children with different NT (Table S2). In general, the numbers of CTCs in CSF were significantly higher than those in peripheral blood, and the numbers of CTCs in tumor patients were significantly higher than those from normal control individuals. Above number of 300 CTC can be found in CSF in anaplastic ependymoma patients, while only bare CTC can be isolated in blood. These results have indicated that cytoplasm GFAP can be selected as an effective CTC separation protein. Of course, the number of CTCs sorted by GFAP-IMLs was related to the age of children, but not associated with tumor stage.

Furthermore, gene mutation detection in separated CTCs by GFAP-IMLs also indicated the tumor-derived circulating cells. Through whole-genome sequencing of DNAs from seven diffuse intrinsic pontine gliomas (DIPGs), 78% of DIPGs contained H3F3A mutations, which was consistent with a previous report [24]. In addition, nearly 80% of DIPGs had the lysine-to-methionine mutation, the famous H3K27M [24, 25], and PCR and Sanger sequencing were used to perform genetic testing on one portion of DIPG patients. H3F3A gene mutations in CSF CTCs and in tumor tissue sample were detected from one of

DIPG patient. What's more, NPR3 overexpression is a characteristic phenomenon in identification of Group 3 Medulloblastoma [26]. The detection of ever-expressed NPR3 in the CTCs from a Group 3 Medulloblastoma was another evidence in supporting the specificity of CTCs isolated by IMLs. NGS method in CTC also indicated the GFAP targeted CTC has tumor features. Indeed, large sample validation still needs to be carried out in our further study.

Conclusions

In summary, the cytoplasm protein GFAP nanoparticles for magnetic separation prepared in the present study effectively isolated CTCs in CSF and peripheral blood from children with neuroepithelial tumors. The constructed GFAP-IMLs separation with DAPI/EGFR-FITC immunofluorescence assay system were effective in the detection of CTCs from CSF and peripheral blood samples. Consequently, this system might be a novel approach providing a valuable reference in the early diagnosis, preoperative and postoperative analysis in children with neuroepithelial tumors and other tumors with cytoplasm protein high expression.

Abbreviations

CSF
Efficient cerebrospinal fluid
CTC
Circulating tumor cell
DIPGs
Diffuse intrinsic pontine gliomas
DOPC
Dioleoylphosphocholine
GHDC
Glycidyl hexadecyl dimethylammonium chloride
HMNs
Hydrophobic magnetic nanoparticles
IMLs
Immunomagnetic liposomes
NT
Neuroepithelial Tumors
PA
Pilocytic astrocytoma

Declarations

Ethics approval and consent to participate

Authors state that they complied with the tenets of the Declaration of Helsinki or the NIH statement, and this study was approved by the Ethics Committee of Xinhua Hospital Affiliated to Shanghai Jiaotong University School of Medicine, all patients involved in this study signed informed consent.

Consent for publication

Not applicable

Availability of data and materials

All data generated and analyzed during this study are included in this published article.

Competing interests

The authors declare that they have no competing interests.

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Authors' Contributions

YZ, FJ and QHW wrote the paper or substantively revised it; BCW, YPH, JY, XSD, JJW made the interpretation of data; YZ, FJ and QHW performed the basic experiments; BCW, YPH, JY, XSD and JJW performed the clinical experiments; KW and JPA performed the cell and animal experiments; XFL and JM have made contributions to the design of the work; XXG, XFL and JM have provide the support of resources. All authors read and approved the final manuscript.

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References

1. Wan JCM, Massie C, Garcia-Corbacho J, Mouliere F, Brenton JD, Caldas C, Pacey S, et al. Liquid biopsies come of age: towards implementation of circulating tumour dna. *Nat Rev Cancer* 2017; 17: 223-238.
2. Shankar, GM, Balaj L, Stott S, Nahed B, Carter BS. Liquid biopsy for brain tumors. *Expert Rev Mol Diagn* 2017; 44:1-5.
3. Guerreiro Stucklin AS, Ramaswamy V, Daniels C, Taylor MD. Review of molecular classification and treatment implications of pediatric brain tumors. *Curr Opin Pediatr* 2018; 30:3-9.
4. Liu KW, Pajtler KW, Worst BC, Pfister SM, Wechsler-Reya RJ. Molecular mechanisms and therapeutic targets in pediatric brain tumors. *Sci Signal* 2017; 10(470).
5. Gajjar A, Bowers DC, Karajannis MA, Leary S, Witt H, Gottardo NG. Pediatric brain tumors: innovative genomic information is transforming the diagnostic and clinical landscape. *J Clin Oncol* 2015; 33:2986-98.
6. Hesham M, Atfy M, Hassan T, Abdo M, Morsy S, Malky ME, Latif D. Pattern of malignant solid tumors and lymphomas in children in the east delta of egypt: a five year study. *Oncol Lett* 2014; 8:2328-2332.
7. Micalizzi DS, Maheswaran S, Haber DA. A conduit to metastasis: circulating tumor cell biology. *Genes Dev* 2017; 31:1827-1840.
8. Neoh KH, Hassan AA, Chen A, Sun Y, Liu P, Xu KF, et al. Rethinking liquid biopsy: Microfluidic assays for mobile tumor cells in human body fluids. *Biomaterials* 2018; 150:112-124.
9. Au SH, Edd J, Haber DA, Maheswaran S, Stott SL, Toner M. Clusters of Circulating Tumor Cells: a Biophysical and Technological Perspective. *Curr Opin Biomed Eng* 2017; 3:13-19.
10. Paoletti C, Cani AK, Larios JM, Hovelson DH, Aung K, Darga EP, et al. Comprehensive Mutation and Copy Number Profiling in Archived Circulating Breast Cancer Tumor Cells Documents Heterogeneous Resistance Mechanisms. *Cancer Res* 2018; 78:1110-1122.
11. Murlidhar V, Reddy RM, Fouladdel S, Zhao L, Ishikawa MK, Grabauskiene S, et al. Poor Prognosis Indicated by Venous Circulating Tumor Cell Clusters in Early-Stage Lung Cancers. *Cancer Res* 2017; 77:5194-5206.
12. Sun YF, Guo W, Xu Y, Shi YH, Gong ZJ, Ji Y, et al. Circulating Tumor Cells from Different Vascular Sites Exhibit Spatial Heterogeneity in Epithelial and Mesenchymal Composition and Distinct Clinical Significance in Hepatocellular Carcinoma. *Clin Cancer Res* 2018; 24:547-559.

14. [El-Heliebi A, Hille C, Laxman N, Svedlund J, Haudum C, Ercan E, et al.](#) In Situ Detection and Quantification of AR-V7, AR-FL, PSA, and KRAS Point Mutations in Circulating Tumor Cells. [Clin Chem](#) 2018; 64:536-546.
15. Adamczyk LA, Hannah W, Aleksandra F, Patricia EH, Haynes HR, Claire P, et al. Current understanding of circulating tumor cell–potential value in malignancies of the central nervous system. [Front Neurol](#) 2015; 6:174.
16. [Schulze K1, Gasch C, Staufer K, Nashan B, Lohse AW, Pantel K, et al.](#) Presence of EpCAM-positive circulating tumor cells as biomarker for systemic disease strongly correlates to survival in patients with hepatocellular carcinoma. [Int J Cancer](#) 2013; 133:2165-71.
17. Kulasinghe A, Tran, THP, Blick T, O’Byrne K, Thompson EW, Warkiani ME, et al. Enrichment of circulating head and neck tumour cells using spiral microfluidic technology. [Sci Rep](#) 2017; 7:42517.
18. Fina E, Reduzzi C, Motta R, Di Cosimo S, Bianchi G, Martinetti A, et al. Did circulating tumor cells tell us all they could? The missed circulating tumor cell message in breast cancer. [Int J Biol Markers](#) 2015; 30: e429-33.
19. [Haber DA, Velculescu VE.](#) Blood-based analyses of cancer: circulating tumor cells and circulating tumor DNA. [Cancer Discov](#) 2014; 4:650-61.
20. [Heller G, McCormack R, Kheoh T, Molina A, Smith MR, Dreicer R, et al.](#) Circulating Tumor Cell Number as a Response Measure of Prolonged Survival for Metastatic Castration-Resistant Prostate Cancer: A Comparison With Prostate-Specific Antigen Across Five Randomized Phase III Clinical Trials. [J Clin Oncol](#) 2018; 36:572-580.
[Magbanua MJM, Hauranieh L, Roy R, Pendyala P, Sosa E, Scott J, et al.](#) Abstract 1591: comprehensive molecular profiling of circulating tumor cells from metastatic breast cancer patients. [Cancer Res](#) 2015; 75:1591-1591.
21. [Zhou Y, Peng Z, Seven ES, Leblanc RM.](#) Crossing the blood-brain barrier with nanoparticles. [J Control Release](#) 2018; 270:290-303.
22. [Magbanua MJ, Melisko M, Roy R, Sosa EV, Hauranieh L, Kablanian A, et al.](#) Molecular profiling of tumor cells in cerebrospinal fluid and matched primary tumors from metastatic breast cancer patients with leptomeningeal carcinomatosis. [Cancer Res](#) 2013; 73:7134-43.
23. [Ding J, Wang K, Tang WJ, Li D, Wei YZ, Lu Y, et al.](#) Construction of Epidermal Growth Factor Receptor Peptide Magnetic Nanovesicles with Lipid Bilayers for Enhanced Capture of Liver Cancer Circulating Tumor Cells. [Anal Chem](#) 2016; 88:8997-9003.
24. [Wu G, Broniscer A, McEachron TA, Lu C, Paugh BS, Becksfort J, et al.](#) Somatic histone H3 alterations in pediatric diffuse intrinsic pontine gliomas and non-brainstem glioblastomas. [Nat Genet](#) 2012; 44:251-3.
25. [Lewis PW, Müller MM, Koletsky MS, Cordero F, Lin S, Banaszynski LA, et al.](#) Inhibition of PRC2 activity by a gain-of-function H3 mutation found in pediatric glioblastoma. [Science](#) 2013; 340:867-61.
26. [Taylor MD, Northcott PA, Korshunov A, Remke M, Cho YJ, Clifford SC, et al.](#) Molecular subgroups of medulloblastoma: the current consensus. [Acta Neuropathol](#) 2012; 123:465-72.

Figures

Figure 1

VSM magnetization curves of the three IMLs. A: Protein electrophoresis. Lanes 1-3 were GFAP, GFAP-GHDC, and GFAP-IMLs, respectively; B: UV spectra. Lines 1-4 indicate the GFAP, GFAP-GHDC, Fe3O4 raw magnetic beads and GFAP-IMLs; C: Raman spectra. Lines 1-3 indicate the GFAP, GFAP-GHDC, and GFAP-

IMLs; D: VSM magnetization curves. Lines 1-3 demonstrate the Fe3O4 raw magnetic beads, magnetic liposomes, and antibody-IMLs, respectively. E: Upper: AFM topographic image and below: particle size distribution of GFAP-IMLs; F: Upper: AFM topographic image and below: particle size distribution of EpCAM-IMLs; G: Upper: AFM topographic image and below: particle size distribution of EGFR-IMLs.

Figure 2

Adhesion and internalization of GFAP-IMLs in U87 cells. Upper to lower panels represent GFAP-IMLs labeled by FITC at different time points (0-30 minutes) in U87 cell plasma, Scale bar: 20 μm.

Figure 3

Verification of the utility of IMLs in the isolation and identification of CTCs in vitro and in vivo. A: Comparison of the cell capture efficiency among the three IMLs in PBS; B: The number of CTCs isolated by three IMLs from mice at different time points after subcutaneous injection (*: $P < 0.05$, **: $P < 0.01$); C: Validation of CTCs captured by IMLs from blood and tumors in nude mice; D: The numbers of CTCs isolated by three IMLs from tumors with different volumes (*: $P < 0.05$, **: $P < 0.01$).

Figure 4

Validation of three IMLs in the isolation of CTCs from patients with pediatric brain tumors. (A-D): Representative fluorescence in cells isolated by GFAP-IMLs from patients' peripheral blood. Isolated cells were labeled with an anti-GFAP monoclonal antibody conjugate by FITC (scale bar: 20 μ m); (E-H): Representative fluorescence in cells isolated by GFAP-IMLs from patients' CSF. Isolated cells were labeled with an anti-GFAP monoclonal antibody conjugate by FITC (scale bar: 20 μ m).

Figure 5

Correlation of isolated CTCs with the clinical information of children with brain tumors. A: Heat map summarizing isolated CTCs from the peripheral blood and CSF of each patient; B: Heat map of CTC isolation from patients with PAs; C: The dot-plot represents the number of CTCs from the peripheral blood and CSF of each patient with PA; D: Heat map of CTC isolation from patients with medulloblastoma; E: Dot-plot of CTCs isolated from the CSF of patients with medulloblastoma. Abbreviations: MB (Medulloblastoma), PA (Pilocytic Astrocytoma), DIPG (Diffuse Intrinsic Pontine Glioma), NC (Normal Control).

Figure 6

A: The number of CTCs captured in CSF with the GFAP, EpCAM, and EGFR magnetic spheres. B: The number of CTCs captured in the blood with the GFAP, EpCAM, and EGFR magnetic spheres. C: The number of CTCs captured by GFAP magnetic spheres in different tumor stages (CSF and blood). D: The number of CTCs captured by GFAP magnetic spheres at different ages (CSF and blood). The average number of CTCs is shown by the horizontal lines. E: The over-expression of NPR3 in CTCs from a patient

diagnosed with Group 3 Medulloblastoma by RT-PCR, which is consistent to the result in immunohistochemistry staining of tumor tissue. (E1: IHC staining of NPR3 in tumor tissue, E2:RT-PCR plot).

Supplementary Files

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