Molecular characterization of cerebrospinal fluid tumor cells associated with leptomeningeal carcinomatosis

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Leptomeningeal carcinomatosis (LC) is a devastating and lethal complication of systemic cancer involving the spread of tumor cells to the meninges and the cerebrospinal fluid (CSF). Efforts focused on molecular analysis of CSF tumor cells (or CSFTCs) are scarce; hence, the biology of these cells remains poorly understood. Our group recently reported a study on molecular characterization of CSFTCs from metastatic breast cancer patients diagnosed with LC. Genomic and gene expression analyses revealed molecular profiles consistent with the malignant nature of CSFTCs. More importantly, we observed a clear clonal relationship between CSFTCs and corresponding primary tumors as demonstrated by high concordance of genome-wide copy number profiles.

Keywords: cerebrospinal fluid; leptomeningeal carcinomatosis; array comparative genomic hybridization; gene expression; fluorescence in situ hybridization analysis


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Introduction

Leptomeningeal carcinomatosis (LC), also referred to as leptomeningeal metastasis, carcinomatous meningitis, or neoplastic meningitis, is a devastating and lethal complication of systemic cancer involving the metastatic spread of tumor cells to the meninges and the cerebrospinal fluid (CSF). Approximately 5-10% of patients with solid tumors, e.g., breast, melanoma, and lung cancer, will develop LC during the course of their disease [1]. The gold standard for LC diagnosis is the detection of malignant cells upon cytological analysis of the CSF [1-2]. However, the detection of tumor cells remains a challenge due to the paucity of cells in the CSF and the difficulty in identifying malignant cells [2]. Although CSF cytology is highly specific, it is not very sensitive. Approximately, 15-20% of cases suspected to have LC in the clinic remain persistently negative after repeated lumbar punctures [3]. LC can also be diagnosed via magnetic resonance imaging (MRI) to ascertain the presence of cranial and/or spinal tumor nodules. More subtle radiographic findings, however, still require confirmation via cytological analysis of the CSF [1].

Patients diagnosed with LC have very poor prognosis. The median survival after diagnosis is about 2 to 3 months [4, 5]. Treatment options are limited to radiation or administration of chemotherapy directly into the CSF [5, 6]. Many challenges are faced in the treatment of LC. This
includes poor penetration of many chemotherapy and biological therapeutic agents to the central nervous system (CNS) [7] as well as the rapid development of resistance to therapy [8, 9]. Additionally, disease in the CNS (both parenchymal brain metastases and LC) is difficult to access and not frequently biopsied, so there is a limited understanding of the biology of metastatic tumor cells that home to these sites.

In this paper, we highlight our recent study on the isolation and detailed genomic profiling of CSFTCs [10]. We also include a brief review of the literature pertaining to genomic analysis of CSFTCs, which has primarily involved fluorescence in situ hybridization (FISH) assays.

**FISH analysis**

Early molecular efforts to characterize CSFTCs involved FISH assays for the detection of copy number alterations of specific chromosome loci. In 1997, van Oostenbrugge and colleagues [11] used FISH to determine copy number aberrations on chromosome 1q12 as evidence for malignant status of cells found by CSF cytology. They examined CSF samples from 22 metastatic cancer patients with cytology-proven LC. Interestingly, FISH analysis revealed that CSF samples from 13 (87%) of the 15 patients successfully analyzed contained malignant cells; whereas control samples from 10 patients with infectious neurological disease (i.e., bacteria or viral meningitis) revealed only benign cells.

A follow-up study compared the sensitivity of FISH analysis vs. conventional cytology and evaluated the utility of each test as a biomarker for response to treatment [12]. Serial CSF samples from 7 patients with known aberrations on 1q12 were aliquoted and subjected in parallel to FISH and cytological analyses. They found that FISH analysis was more sensitive than conventional cytology in detecting malignant cells. More interestingly, the FISH results correlated better with patients’ neurologic status, i.e., more malignant cells were found in the CSF of patients whose neurological symptoms continued to deteriorate despite intrathecal chemotherapy.

One recent study compared the HER2 status of primary tumors vs. CSFTCs in 16 metastatic breast cancer patients with LC [13]. HER2 status in primary tumors was assessed by immunohistochemistry (IHC) and confirmed by FISH analysis of HER2 (chromosome 17q12) in cases where IHC results were equivocal. Using the same FISH assay, HER2 status of CSFTCs on previously stained CSF cytology slides was analyzed. HER2 status was noted to be highly concordant (94%) between CSFTCs and corresponding primary breast tumors.

**Isolation of CTCs and CSFTCs via IE/FACS**

Our group recently described a new method for the efficient isolation of circulating tumor cells (CTCs) from blood of metastatic breast and prostate cancer patients [14, 15]. The isolation technique is a two-step process consisting of immunomagnetic enrichment followed by fluorescence activated cell sorting (referred to as IE/FACS) [16]. In the initial step, magnetic beads coated with monoclonal antibodies to the epithelial cell adhesion marker (EPCAM) were used to enrich for tumor cells. The second step involves FACS analysis of the tumor-enriched sample using differentially labeled monoclonal antibodies to isolate tumor cells (EPCAM+) away from leukocytes (CD45+).

We subsequently applied this general isolation strategy to obtain populations of tumor cells and leukocytes (as non-tumor control) from the CSF of metastatic breast cancer patients with diagnosed or suspected LC. We subjected the IE/FACS-isolated cells to different molecular assays, including gene expression and copy number analysis [10].

**Gene expression analysis**

CSFTCs in serial CSF samples (n=18) from 5 patients
were successfully isolated and analyzed by multiplexed quantitative PCR of 64 cancer-related genes. Unsupervised hierarchical clustering analysis revealed that CSFTC gene expression profiles were clearly distinct from leukocytes attesting to the high purity of cell isolation via IE/FACS. As expected, we observed significant up-regulation of...
EPCAM and down-regulation of PTPRC (encodes CD45) expression (Figure 1). In addition, we also observed significant up-regulation of AGR2, TFF3, and GRB7 in CSFTCs (Figure 1). Over-expression of these genes is associated with aggressive breast cancers. For example, AGR2, a target of the estrogen receptor, has been shown to promote breast cancer metastasis (reviewed in [17]). Over-expression of TFF3 [18] and GRB7 [19, 20] is associated with highly metastatic breast cancer phenotype with poor prognosis.

**Genome-wide copy number analysis**

Next, we subjected CSFTCs isolated from 13 patients to array comparative genomic hybridization (ACGH) analysis to examine genome-wide copy number aberrations. CSFTCs displayed genomic aberrations consistent of breast cancer origin, including gains on 1q and 8q, and losses on 1p and 8p. Additionally, the magnitude of the apparent copy number changes indicated little or no evidence of hematopoietic cell contamination. Overall, we observed similar recurrent aberrations when CSFTC profiles were compared to those of CTCs and primary breast tumors (Figure 2A). Interestingly, CSFTCs exhibited high-level gains on chromosome 8q24 containing the MYC oncogene (see example in Figure 2B).

Furthermore, comparison of genomic profiles of CSFTCs vs. matched archival primary tumor revealed similar patterns of copy number alterations indicating clonal-relatedness (Figure 2C). Genomic alterations, however, were more extensive in CSFTCs suggesting higher purity as compared to primary tumor samples, which can be contaminated with non-tumor cells (e.g., stromal cells). It is also possible that CSFTCs may have acquired further alterations during disease progression. We also compared the HER2 status between CSFTCs assessed by ACGH (see example in Figure 2B) and corresponding primary tumors evaluated either by IHC/FISH and ACGH and found a high agreement (83%) consistent with previous report [13]. In one patient, CSFTCs from serial samples were collected (n=5) and subjected to ACGH analysis. Good concordance of genomic profiles of tumor cells demonstrated the reproducibility and feasibility of longitudinal molecular analysis of CSFTCs using our method (Figure 2D).

**Summary**

Very little is known about leptomeningeal carcinomatosis and the cancer cells responsible for this dreaded syndrome. Early molecular studies on CSFTCs using FISH showed the feasibility of detecting specific copy number aberrations. Recently, we reported the feasibility of efficiently isolating CTCs for detailed molecular analyses. We have adapted this strategy to capture and profile CSFTCs from patients with known or suspected LC. It is therefore now possible to characterize CSFTC using the same modern molecular techniques being applied to primary tumors, including genome-wide copy number analysis as well as gene expression profiling. We anticipate future studies using further approaches such as next generation sequencing of CSFTCs.

Understanding the genetic relationship between CSFTCs and corresponding primary tumors may shed new light on the mechanisms involved in tumor evolution and cancer progression. For example, we observed a high concordance of genome-wide copy number alterations in addition to HER2 copy number status between CSFTCs and corresponding archival primary tumors, confirming clonal-relatedness and suggesting continued selection of oncogenic drivers.

It will also be particularly important to explore CSFTC-associated genomic alterations, which may provide novel insights into tropism of tumors to the CNS. Identification of molecular changes and signatures specific to CSFTCs may lead to the discovery of new prognostic biomarkers and targets for therapy.

**Conflicting interests**

The authors have declared that no competing interests exist.

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