Discovery of Cancer Stem Cell Biomarkers Using Metabolomics

Paul R. West, Ph.D., Alan M. Smith, Ph.D. , April M. Weir, M.S., Gabriela G. Cezar, DVM, Ph.D.

Stemina Biomarker Discovery, Inc., 504 S. Rosa Rd, Suite 150, Madison, WI 53719

Overview

- Treat brain tumor cells, cancer stem cells and normal neural cells with anti-cancer drugs and radiation.
- Perform HILIC-QTOF-MS nontargeted metabolomic analyses
- Discover small molecules unique to brain tumor stem cells for future use as biomarkers for drug and radiation therapy efficacy.

Introduction

Glioblastoma multiforme (GBM) is one of the most malignant forms of cancer with a mean survival time of one year (1). Brain tumor stem cells (BTSCs), have successfully been isolated and are believed to be the cause of GBM tumor recurrence, are highly resistant to therapies (2,3). Our objective was to discover secreted small molecules that are unique to, or elevated in, BTSCs and not found in GBM tumor cells or in neural stem cells (hNSCs), then to study the levels of these biomarkers in response to various treatments in order to assess treatment efficacies against BTSCs in vitro. These small molecules can potentially be used to monitor treatment regimens and serve as a basis for high throughput assays measuring the efficacy of new drug and radiation therapies against the recalcitrant BTSCs.

Methods

In order to find BTSC biomarkers of drug efficacy, our first step was to determine the small molecule metabolites that are common amongst three different BTSC lines. This set of BTSC biomarkers was compared to non-CSC, differentiated cells in glioblastoma multiforme (GBM) tumors and human neural stem cells (hNSCs). As a result, comparative metabolomics provided a means to identify the BTSC-specific biomarkers that are not present in these other two cell lines. These small molecules can be used to monitor different GBM therapeutic modalities or to perform screening of chemical libraries in order to determine the efficacy of such small molecules or biologicals against BTSCs.

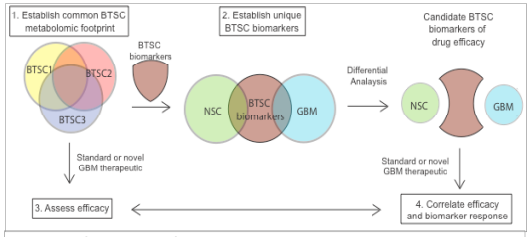

Figure 1. Schematic of experimental design.

Cell Culture

The John S. Kuo laboratory supplied the cells under study: three BTSC lines, three GBM lines, and one hNSC line. Cell culture procedures were performed in their laboratory at the University of Wisconsin School of Medicine and Public Health. BTSC lines and the hNSC lines were cultured and formed neurospheres in T25 suspension flasks. The BTSC lines were individually characterized according to cell surface antigens indicative of cancer stem cells phenotype (CD133), as well as their multipotentiality, or ability to differentiate into neurons and astrocytes in a similar manner as neural stem cells (Figure 2). Flasks of neurospheres and hNSCs were transferred to six-well plates for experiments. GBM cells were cultured in six-well plates at a density of 50,000 cells per well for experiments. For each cell type, 12 six-well plates were prepared: six treated plates and six control plates (Figure 3). Also, 96-well plates were prepared for MTSC assays. Cells were seeded and dosed two days afterwards. Spent medium was collected four days following dosing and prepared for mass spectrometry analysis.

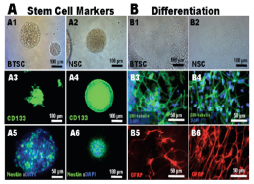

Figure 2.
BTSC immunohistochemistry

Treatments

All cell lines underwent the following treatments (Figure 3): control, irradiation, debromohymenialdisine (DBH), temozolomide (TMZ), radiation + DBH, and radiation + TMZ. Radiation + DBH treatment was found to be the only treatment to which the BTSCs were resistant.

Sample Preparation

Spent medium samples were collected and quenched with 40% acetonitrile to both halt metabolic processes and precipitate proteins. Samples were filtered using a 3KDa molecular weight cut-off column (Microcon, Millipore) then dried in a SpeedVac and, lastly, reconstituted in 50 µL 0.1% formic acid.

Mass Spectrometry

Liquid chromatography electrospray ionization quantitative time of flight mass spectrometry (LC-ESI-QTOF-MS) was performed on an Agilent system with a Phenomenex Luna HILIC 100 x 3mm, 5µm column (Figure 4). Data acquisition was performed with Agilent Masshunter version B.01.03 using high resolution exact mass conditions.

Statistical Analysis

Mass features were binned based on retention time and mass in order to consider a mass feature the same across MS runs. Mass features were then filtered and accepted if it was present in at least 60% of the sample replicates and present at abundance levels of at least 30% above or below the media. Boolean logic was used to identify features unique to the BTSC samples and not present in the non-BTSC lines.

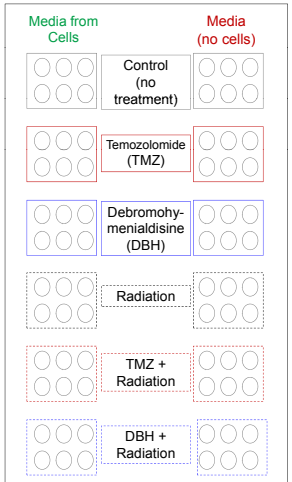

Figure 3. Illustration of experiment design per cell line.

Figure 4. HILIC gradient conditions.

Flow rate:	0.5 mL/min.
Solvent A:	0.1% Formic Acid in water
Solvent B:	0.1% Formic Acid in ACN

Time	%A	%B
0.0	5.0	95.0
1.5	5.0	95.0
16.0	40.0	60.0
17.0	95.0	5.0
21.0	95.0	5.0
22.0	5.0	95.0
30.0	5.0	95.0

Results

We first analyzed the three BTSC lines: BTSC 12.1, 22, and 33 for mass features that were common among the three independent lines. These 456 features represented the BTSC secretome (Figure 5). This BTSC secretome was then compared to the secretomes of GBM tumor cells and hNSC to remove features not specifically related to BTSCs. This created a subset of features representing the unique metabolomic footprint of BTSC cells. When analyzing the different cell types, we found clear differences between the cell types, as illustrated in the principle component analysis (PCA) plot (Figure 6).

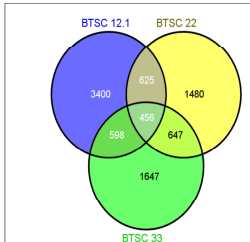

Figure 5. Venn diagram showing common features between the three different BTSC lines.

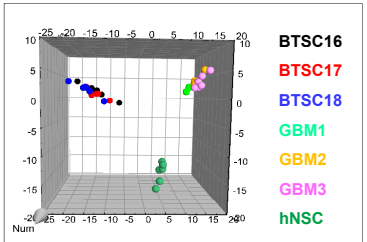

Figure 6. Principle component analysis (PCA) plot of BTSC lines, GBM lines, and the hNSC line.

There is clear evidence that the secretomes between these lines are distinguishable. We have found 64 candidate BTSC biomarkers (mass features unique to BTSCs) in HILIC ESI(+) mode data and 67 in HILIC ESI(-) mode data (Figure 6). The mass features unique to BTSCs are candidate biomarkers of BTSCs and are currently being validated and studied. After finding BTSC candidate biomarkers, we wanted to determine which of these could potentially serve as biomarkers of efficacy. We looked for mass features that had significantly altered abundances only in the IR + DBH conditions and unchanging abundances in all other conditions. We found 6 candidate biomarkers of efficacy. The chemical structures of 2 of these compounds have been validated and the remaining 4 are under investigation and validation.

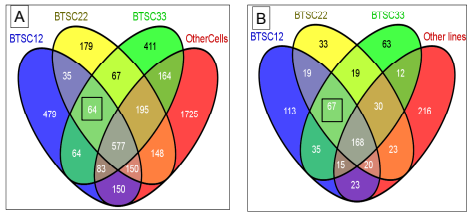

Figure 6. Venn diagram of mass feature bins used to analyze the different treatment regimens and to select the unique BTSC metabolomic footprint. The red square marks the intersection containing features unique to the 3 BTSC lines, but not present in the hNSC line or the three GBM cell lines.
A. ESI (+) feature bins. **B.** ESI (-) feature bins.

Conclusions

Stemina has accomplished several milestones under this contract study. We have found small molecules that are unique to GBM cells, which can serve as screening molecules for GBM. We have also found unique BTSC small molecules that are common amongst three various BTSC lines. These have the potential to provide a sensitive and accurate method of measuring GBM presence and progression. Both GBM and BTSC biomarkers may also serve as therapeutic targets in order to manufacture drugs that are targeting GBM and BTSC cells. Lastly, we have discovered a subset of these unique BTSC small molecules that are candidate biomarkers of drug efficacy, two of which the chemical identities have been confirmed. Such biomarkers can be used to assess novel drugs' efficacies against BTSCs, specifically, which will help lead drug developers to more powerful therapeutics that have the potential to prevent tumor recurrence. Further work is being performed to gain a deeper understanding into BTSC biology and behavior.

Current efforts are being made to validate more biomarkers of efficacy. Stemina also intends to perform an in vivo study to further validate biomarkers of efficacy and to develop biomarkers that can be detected in human biofluids.

References

1. Hide T., et al. Brain Tumor Pathology 2008; 25(2): 67-72.
2. Singh S.K., Clarke I.D., Terasaki M., et al. Cancer Res 2003; 63(18): 5821-8.
3. Liu J.M., Mao B.Y., Hong S., et al. Adv Ther 2008; 25(5): 389-98.
4. Bao S., Wu Q., McLendon R.E., et al. Nature 2006; 444(7120): 756-60.

Acknowledgements

We would like to acknowledge Dr. John S. Kuo and his laboratory, including Sasha Rackman and Priya Ezhilan. We are also grateful to Agilent technologies for their support and equipment.

Funding

This work was funded by NIH/NCI SBIR Contract Number: HHSN261200800024C

www.stemina.com