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Cancer Stem Cells in the Screening of Anticancer Drugs for Central Nervous System Tumors

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CANCER STEM CELLS IN THE SCREENING OF ANTICANCER DRUGS FOR CENTRAL NERVOUS SYSTEM TUMORS

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DEDICATION

I wish to dedicate this dissertation:
To my beautiful daughter Madeleine Josephine Daron-Mathis.
You are my hope.
To my loving husband John Mathis.
You are my strength.
To my Aunt Madeleine and Uncle Okie.
You are my foundation.

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LIST OF ABREVIATIONS

2D	Two Dimensional
3D	Three Dimensional
ABCG2	ATP-binding cassette sub-family G member 2
AMP	Adenosine monophosphate
ANOVA	Analysis of variance
APC	Allophycocyanin
Ara-C	Arabinoside-C
ATP	Adenosine triphosphate
AVMA	American Veterinary Medical Association
BBB	Blood Brain Barrier
BCNU	Carmustine
BITC	Benzyl isothiocyanate
BPC	Biopathology Center
BSA	Bovine Serum Albumin
BUS	Busulfan
CCNU	Lomustine
CD	Cluster of differentiation
CDDP	Cisplatin
CLIA	Clinical Laboratory Improvement Amendments
CNS	Central Nervous System
COG	Children's Oncology Group

CPL	Carboplatin
CPT-11	Irinotecan (Camptosar)
CSC	Cancer stem cell
CSLC	Cancer stem-like cell
CT	Computed Tomography
CTX	Cyclophosphamide
CXCR4	Chemokine Receptor Type 4
CyQUANT	CyQUANT® Cell Proliferation Assays
DACH	Dilute-Acid Cellulose Hydrolysis
DHFR	Dihydrofolate reductase
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribose Nucleic Acid
EDTA	Ethylene diamine tetra acetic acid
EGFR	Epidermal Growth Factor Receptor
FACS	Fluorescence activated cell sorting
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
FSC-A	Forward Scatter-Area
GBM	Glioblastoma Multiforme
GCB	Gemcitabine
GFAP	Glial fibrillary acidic protein
HFB	Hydrodynamic Focusing Bioreactor

IACUC	Institutional Animal Care and Use Committee
IBM SPSS	International Business Machines Statistical Package for the Social Sciences
IDH-1	Isocitrate dehydrogenase 1
i.p.	Intraperitoneal injection
IRB	Institutional Review Board
ITC	Isothiocyanates
KRAS	Kirsten rat sarcoma viral oncogene homolog
MACS	Magnetic-activated cell sorting
MGMT	O 6-methylguanine DNA methyltransferase
MRI	Magnetic resonance imaging
MTT	Methyl-Thiazolyl-Tetrazolium
MTX	Methotrexate
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate - hydrogen
NCCN	National Comprehensive Cancer Network
NGS	Next Generation Sequencing
NOD-Scid	Non-obese diabetic-Severe combined immunodeficiency
NSCLC	Non-small cell lung cancer
OCT	Octamer transcription factor
OXA	Oxaliplatin
PAS	Positive fibrillar material
PBS	Phosphate Buffer Solution
PCB	Procarbazine

PE	Phycoerythrin
Prom-1	Prominin-1 Protien
RBC	Red Blood Cell
RNA	Ribonucleic acid
ROS	Reactive Oxidative Species
RPMI	Roswell Park Memorial Institute
SSC-A	Side Scatter-Area
TMZ	Temodar
VEGF-A	Vascular Endothelial Growth Factor A
VCR	Vincristine
VP-16	Etoposide
WHO	World Health Organization
WST-8	Water-soluble Tetrazolium
Wt	Wild type

ABSTRACT

There is a growing need (in the medical field) to design personalized therapy for cancer patients. Decades of cancer research have found no silver bullet that can cure all or even most patients. This study evaluated four patients affected by central nervous system (CNS) tumors (Ependymoma and Glioblastoma), and found that tumors with the same histology had unique responses to treatment. Each sample presented different levels of heterogeneity in expressed biomarkers and responded to drugs at varying levels.

Oncologists conventionally treat cancer patients with drugs tested in large clinical trials. However, often patients do not experience positive outcomes following treatments with standard-of-care first line drugs and oncologists need to treat them with a different second-line anticancer therapy that is chosen empirically. This study was designed to find a way to better predict patient's response to chemotherapeutic drugs. The focus of this study was on Central Nervous System (CNS) tumors because of their limited response to anticancer drugs and their low survival rate. The uniqueness of this study revealed that each patient's tumor had different drug sensitivities and that screening for multiple drugs may increase the chance of finding a drug from which the patient would have the most benefit.

More importantly this study evaluated the Cancer Stem-Like Cell (CSLC) population sensitivity to these drugs. This subpopulation is responsible for initiation and maintenance of the tumor and is known to be resistant to chemotherapy drugs. Dr. Claudio's laboratory developed a test capable of determining the cytotoxic drug to which cancer cells and CSLCs of an individual tumor are most responsive. In the future this procedure may focus the treatment of CNS patients to drugs effective against their particular tumor allowing them to have better outcomes with fewer detrimental side effects.

CHAPTER 1: DEVELOPMENT OF A PERSONALIZED CHEMOTHERAPY ASSAY

INTRODUCTION

The typical treatment for most cancer is surgery followed by chemotherapy and/or radiation therapy combined in various modalities (1). Ordinarily, the type of chemotherapy or amount used is based on histology, clinical characteristics of the patient, and retrospective evidence from randomized clinical trials (2). The standard approach for the treatment of brain cancer is also surgery followed by chemoradiation therapy. However; the chemotherapy choices are more limited due to the Blood Brain Barrier (BBB), which may limit diffusion of the drugs from the blood stream to the nervous tissue (3,4). There are several CNS tumor types and overall for all types of malignant brain tumors in adults, around four out of ten people diagnosed (40%) live for at least a year. About 19 out of every 100 people (19%) live for at least five years after diagnosis, and around 14 out of every 100 people diagnosed (14%) live for at least ten years. Unfortunately 50% of all CNS cancers diagnosed are Glioblastoma Multiforme (GBM), which shows the lowest survival rate of all, with survival only being 12-18 month after diagnosis (3). With such a short survival time, the patient rarely has a second or third round of treatment.

Oncologists cannot predict if a chemotherapeutic agent will be effective for a patient beforehand, all they can determine is that a particular drug has been effective in a percentage of patients either from their own experience or from large randomized clinical studies (2). Patients with the same tumor histotype often respond differently to the same chemotherapy regimen due to heterogeneity of the tumor (2). Following an initial treatment with drugs used as first-line chemotherapies, the treating physician will be able to determine if the patient is responding or not to a particular chemotherapy regimen. Tumor relapse may still occur in spite of an initial

response because of the presence of a subset of cells that are resistant to the treatment (5). Toxic side effects may also decrease patients' quality of life. Most chemotherapies impact several important functions and organs of the human body that have cells undergoing a rapid turnover by having active cellular divisions, such as the hematopoietic compartment, the colon, and the hair follicles thereby causing immune depression, diarrhea, and hair loss. Additionally, chemotherapy may also diminish the patients' memory and learning abilities (6). Therefore, it would be advantageous to both the patient and the doctor to know whether a chemotherapeutic agent will be effective against the patient's tumor cells. This approach would increase efficacy against the tumor, reduce toxicity and side effects for the patient, prolong response, and reduce medical cost.

Because of these concerns many different scientists have been looking into therapeutic or diagnostic approaches to personalize patient therapy. Next Generation Sequencing (NGS) aims at determining genomic markers that would predict the patient's sensitivity to a specific chemotherapeutic agent (7). Evidence linking genetic information to therapeutic effectiveness of treatments can be misleading. Following the discovered association of KRAS mutations with resistance to anti-EGFR antibodies, some studies have shown that tumors with normal 'wild-type' KRAS profile, still do not respond to therapy (8). This type of research is still in its infancy and needs more time to progress as research furthers the understanding of tumor biology.

Other researchers have developed a method to determine the sensitivity of cancer cells to chemotherapeutic agents (total cancer cell population). However, their studies show no accurate and statistically significant correlation to patients' disease outcomes (9-12). In the present study the developed method for personalized chemotherapy uses a biopsy from the patient, which

allows for testing of the total cancer population and also the CSLCs against standard-of-care chemotherapy drugs.

CNS Cancer

There are several types of tumors of the central nervous system (CNS); however CNS tumors are only 1.4% of all new cancers diagnosed in the United States (13) (SEER Stat Fact Sheets: Brain and Other Nervous System Cancer. http://seer.cancer.gov/statfacts/html/brain.html). Gliomas are typically found in adult cases and are fast growing brain tumors typically arising from the supportive tissue of the brain: the "glia". Three types of normal cells that constitute the glia can become cancerous. Astrocytes will form astrocytomas, including glioblastoma. Oligodendrocytes will transform into oligodendrogliomas, and ependymal cells form ependymomas (13,14). Gliomas are further classified by the World Health Organization (WHO) according to their grade. Low grade gliomas (WHO grade II) are well differentiated tumors that tend to have better prognoses. High-grade gliomas (WHO grade III Astrocytoma, WHO grade III Ependymoma, and WHO grade IV glioblastomas) are undifferentiated (anaplastic) and carry the worse prognosis. The highly malignant anaplastic gliomas display hypercellularity, nuclear atypia, and high mitotic activities at histopathologic examination (13,14). This study focused on high-grade Astrocytomas known as Glioblastoma Multiforme (GBM) and high-grade Ependymoma.

Standard-of-Care Drugs

Several factors were taken into account when determining what drugs would be used for this study. 1. NCCN guide lines, 2. Treating Physician and Pharmacist consultation, 3. Availability of drug. 4. Mechanism of drugs cytotoxicity. All mechanisms of action for drugs used were reviewed to ensure cytotoxicity was a measurable endpoint.

Arabinoside-C (Ara-C), also known as Cytosine Arabinoside, interferes with the synthesis of DNA by rapidly conversion to Cytosine Arabinoside triphosphate, which damages DNA during DNA synthesis, and also inhibits both DNA and RNA polymerases and nucleotide reductase enzymes needed for DNA synthesis (15).

Bevacizumab (Avastin) is a monoclonal antibody, its mechanism of action is to block angiogenesis by inhibiting vascular endothelial growth factor A (VEGF-A) (16). The antibody stops the receptor from being activated and cuts off any new blood supply to the growing tumor, causing the growth to stop (17). However, this drug has been known to also have cytotoxic effects but the mechanism is still debated (18). VEGF-targeted therapies, such as bevacizumab, exert their effects through a number of potential mechanisms, including (1) inhibition of new vessel growth, (2) regression of newly formed tumor vasculature, (3) alteration of vascular function and tumor blood flow ("normalization"), and (4) direct effects on tumor cells by activation of apoptosis (19).

Busulfan (BUS) is an alkylating agent that forms DNA-DNA crosslinks between the DNA bases guanine and adenine and between guanine and guanine which prevents DNA replication (20). These DNA crosslinks cannot be repaired, so the cells undergo apoptosis.

Carboplatin (CPL) is a second generation platinum drug (21). Two mechanisms of action have been proposed: the aquation hypothesis and the activation hypothesis (22). The aquation

hypothesis proposes that the drug complex aquates and reacts with DNA like cisplatin (22,23). The difference is in the rate of activity between the faster Carboplatin and Cisplatin (24). The activation hypothesis assumes that Carboplatin is biologically activated and that it is an unknown platinum species that binds to the DNA (22). Carboplatin requires a much lower and more tolerable dose that is then activated within the cell itself (22).

Carmustine (BCNU) is a mustard gas compound used as an alkylating agent in chemotherapy (25). It disassociates to yield 2-chloroethyl isocyanate and the chloroethyl carbonium ion intermediate, which is the alkylating moiety (25). As an alkylating agent, BCNU is able to form interstrand crosslinks in DNA; thereby DNA cannot replicate or be transcribed (25).

Cisplatin (CDDP) is a platinum drug that causes DNA intrastrand cross-links between two adjacent guanine residues on the same DNA strand (6,21,23). Patients that have overexpression of ABCG2 and other multidrug resistance are not responsive to CDDP (6). It is of note that CDDP causes severe toxicity, which has led to the development of several second generation platinum drugs (21,26).

Topoisomerase II is a primary cellular target for Etoposide (VP-16) (27). Without topoisomerase II cells cannot resolve the knots and tangles in the genetic material that are produced by normal cellular processes (27). Etoposide inhibits topoisomerase II and in its absence the cells are unable to replicate DNA and the cells die as a consequence of mitotic failure (27).

Irinotecan (Camptosar, CPT-11) is a topoisomerase-I inhibitor, which is a nuclear enzyme associated with relaxation of the supercoiled DNA (28,29) that produces reversible single-strand breaks in DNA during DNA replication. These single-strand breaks relieve

torsional strain and allow DNA replication to proceed. Irinotecan binds to the topoisomerase I-DNA complex and prevents religation of the DNA strand, resulting in double-strand DNA breakage and cell death (29).

Lomustine (CCNU) is another DNA alkylating agent (30,31). Lomustine's full mechanism of action has not been fully understood but more recent studies have shown that CCNU triggers apoptosis through the intrinsic apoptosis mitochondrial pathway (31). Its mechanism of action also involves the inhibition of both DNA and RNA synthesis through DNA alkylation. Lomustine has been shown to affect a number of cellular processes including: RNA and protein synthesis; the processing of ribosomal and nucleoplasmic messenger RNA; DNA base component structure; the rate of DNA synthesis and DNA polymerase activity. It is cell cycle nonspecific.

Methotrexate (MTX) binds to and competitively inhibits Dihydrofolate Reductase (DHFR) an enzyme that participates in tetrahydrofolate synthesis (32). Without this reaction the biosynthesis of purines, thymidylate, and several amino acids is inhibited and the cells cannot replicate or repair DNA (32).

Oxaliplatin (OXA), like the other platinum drugs causes intrastrand adducts. Oxaliplatin belongs to the 1,2-diaminocyclohexane (DACH) carrier ligand family whereas cisplatin and carboplatin belong to cis-diammine. Oxaliplatin is a bulky hydrophobic DACH ligand, which prevents binding of DNA repair proteins (23). This was the first drug approved that could overcome cisplatin resistance (23,26).

Procarbazine (PCB) has multiple mechanisms of action (33). It inhibits incorporation of small DNA precursors, as well as inhibits RNA and protein synthesis (33). PCB can also directly

damage DNA through an alkylation and methylation reaction (33,34). It is almost always administered in combination with CCNU and VCR (33,35).

Temodar (TMZ) is commonly used as a first-line drug for high grade gliomas (6). TMZ is a DNA-alkylating agent and methylating agent (6,35) which delivers a methyl group to purine bases of DNA (O6-guanine; N7-guanine and N3-adenine). This methylation of guanine residues leads to single and double-strand DNA breaks and subsequent apoptotic cell death.

Vincristine (VCR) is an alkaloid derived from *Vinca rosea* Linn (36). It is known to disrupt mitosis by interfering with microtubules. Like other vinca alkaloids, Vincristine may also interfere with: 1) amino acid, cyclic AMP, and glutathione metabolism, 2) calmodulin-dependent Ca2+-transport ATPase activity, 3) cellular respiration, and 4) nucleic acid and lipid biosynthesis (30,37).

Cancer Stem Cell Theory

Dr. John Dick is credited with the discovery of Cancer Stem-Like Cells. The Cancer Stem-Like Cell theory describes cancer stem-like cells (CSLCs) as a subpopulation that initiates and maintains cancer, allowing for recurrence and therapy resistance (38-40). However, since then the Cancer Stem-Like Cell theory has been one of controversy (41). “This controversy developed because “the definition” of a cancer stem cell needs to be linked to the functional assay that is used to identify it. Also it needs to be clearly understood that the “cell of origin” (stem cell or not) represents a different issue” according to Dr. John Dick (42).

The consistent question is whether a cancer always originates from normal stem cells which lose the control of proliferation and differentiation, or is it a differentiated cell that becomes more stem-like after acquiring mutations (42-44). Defining CSLCs as a subpopulation

of tumor cells able to develop their cancer tissue type and regenerate a phenotypically heterogeneous cell line, like that of the original tumor, implies that CSLCs are the driving force of cancer recurrence (5). The difficulty to experimentally address this question arises from the limited quantity of this subpopulation of CSLCs in a given tumor (43). This may one day be addressed by the ability to isolate and enrich this population (45).

This study evaluates CD133+ subpopulation of CNS tumors for isolation and testing. CD133 is also known as Prominin-1 (Prom-1), a pentaspan membrane glycoprotein (46). CD133 was first identified as a hematopoietic stem cell marker and since then has been shown to be a marker of cancer stem-like cells with prominent tumorigenic potential (46). Its function is not fully understood but is believed that in tumor cells it regulates the proliferation and colony-formation of cancer cells (6, 45-50). CD133 has also been associated with CSLC population in many tumor types including Glioblastoma, Ependymoma, Medulloblastoma, and Meningioma (47-49). The role of CD133 as a CSLC marker has been actively investigated over the years (46). It is accepted that glioma stem-like cells have high expression of CD133 that is similar to neural stem/progenitor cells (6,46).

Clinical Study

The clinical study using the chemosensitivity assay was set up with the following steps: 1. Receive the biopsy. 2. Disassociate and grow the cells in a monolayer. 3. Determine the biomarkers that identify CSLCs. 4. Treat bulk cells with various chemotherapeutic agents. 5. Expand CSLCs and treat them with various chemotherapeutic agents. 6. Analysis of chemosensitivity data and correlation to clinical outcome.

The tumor biopsies from the CNS patients were received in a transfer/collection media tube. This consisted of RPMI and 10% FBS with 4% Penicillin/Streptomycin. During surgery a piece of the tumor was collected and placed in this collection tube, which allowed time for any introduced contamination to be exposed to high levels of antibiotic, reducing the risk of growth of contaminant bacteria. Culture viability tests were run to show that the tissue remained viable for several days following collection at room temperature (Figure 1).

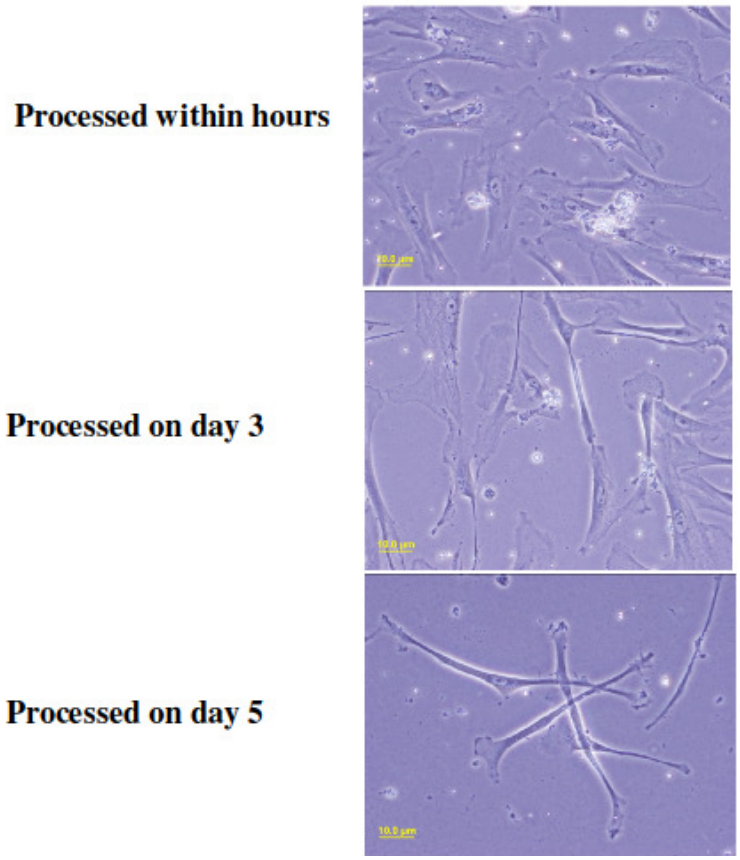

Figure 1. CNS Tumor collected and processed at different times after collection.

Once biopsy was received tissue was cut into three pieces. Top: Tissue was processed immediately and incubated for five days. Middle: Tissue was left in test tube with transfer/collection media for three days at room temperature then processed and incubated for five days. Bottom: Tissue was left in test tube with transfer/collection media for five days at room temperature then processed and incubated for five days.

Once received by the lab the tissue was washed thoroughly. If the received tissue was bloody, then it was washed with PBS several times in attempt to reduce the amount of Red Blood Cells (RBCs) that would be in the culture. After this initial washing, one more wash with 100% Penicillin/Streptomycin was conducted before manual disassociation technique. To manually disassociate the tissue a sterile scalpel was used to mince the tissue. The minced tissue was collected into a 15 mL sterile tube with equal amounts of 0.25% Trypsin and 0.1uM EDTA

Accutase. The volume of trypsin and accutase mixture varied based on the amount of tissue received. The tube was then placed in a 37°C water bath for 5-15 minutes (until the solution looked cloudy) and then the tissue was transferred into a tissue culture treated dish and normal culture media was added to stop the digestion (45,50). The primary culture was observed using a bright field inverted microscope every day for about two weeks and then a regular sub culture technique began (45,50). Once several tissue culture treated dishes were sub-cultured, several stocks were made and cryopreserved.

To determine presence of CSLCs in the primary culture obtained, flow cytometry using fluorescent antibodies against known CSLC markers were used (3,6,38,40,45-55). For flow cytometry analysis, $1x10^6$ primary cells were collected, stained using specific antibodies and analyzed using an Accuri C6 Flow Cytometer (45,50). Unspecific isotype antibodies were used as controls. The isotype control is a base antibody with just the unspecific clone that has a fluorescent marker added; it detects unspecific binding to remove any false positives from the test. Expression of every marker tested was calculated by subtracting the value calculated from sample stained with the isotype antibody. This initial testing is important to verify that the CSLCs are enriched in the following steps of the test.

The amounts of drugs were based on the clinical dose. The equations used were: Stock concentration $(mM) = (concentration\ of\ drug/m.w.) * 1000$, *In vitro* dose$(mM) = (clinical\ dose\ (mg/m^2) * 1.6)/m.w.$, Amount of stock needed to treat cells $= (In\ vitro\ dose/stock\ concentration) * 1000$ = ul of stock/ml of media. This concentration was added to the culturing medium to treat the cells for a one hour pulse to mimic the highest concentration of chemotherapy drug to which cancer cells would be exposed in a treated patient.

Medium was then removed and cells were fed with complete medium containing 10% FBS for 24 hours and assayed using an MTT assay.

Methyl-Thiazolyl-Tetrazolium (MTT) proved to be an accurate assay for determining cell viability for this chemotherapy sensitivity assay (56). MTT is a yellow powder called (3-(4, 5-dimethylthiazole-2-yl)-2, 5-diphenyltetrazoli-umbromide) that is reduced by mitochondrial dehydrogenase in viable cells, forming a purple formazan precipitant (56). After a 2-4 hour incubation the media with MTT is removed and the precipitant can then be solubilized using 50ul of DMSO per well. Once the 96 well plate is briefly shaken the plate can be read on a spectrophotometer at 550nm absorbance. The readings provide the absorbance of the control and each of the treatments, by taking the (mean of the treatments/means of the controls)*100= % viable compared to control. This assay is widely used to assess cell viability after imitation of cytotoxicity (57), however this has been challenged in recent years. One reason that this is challenged is because MTT can react with compounds used in treatment. This was avoided by mixing the chemotherapeutic agents used with the MTT and evaluating the absorbance. No change was found (data not shown). The other challenge with using the MTT assay is that not all cells have the same amount of mitochondrial activity which is where the MTT is enzymatically changed into the purple formazan percipient. This is especially a problem with established cell cultured lines. It was found in primary lines to be less of a challenge because less genetic shifts have happened. However, a comparison of the MTT assay to several other types of viability assays (CyQUANT, WST-8, and ATP luciferase) was performed on the primary cultures and found that there was no statistical difference among the different types of assays tested (Figure 2). In particular, the WST-8 assay was found to be a more reliable alternative if cells were not

attaching to the surface of the 96-well dishes because WST-8 is water soluble and it doesn't require washing steps that are necessary in an MTT assay (58).

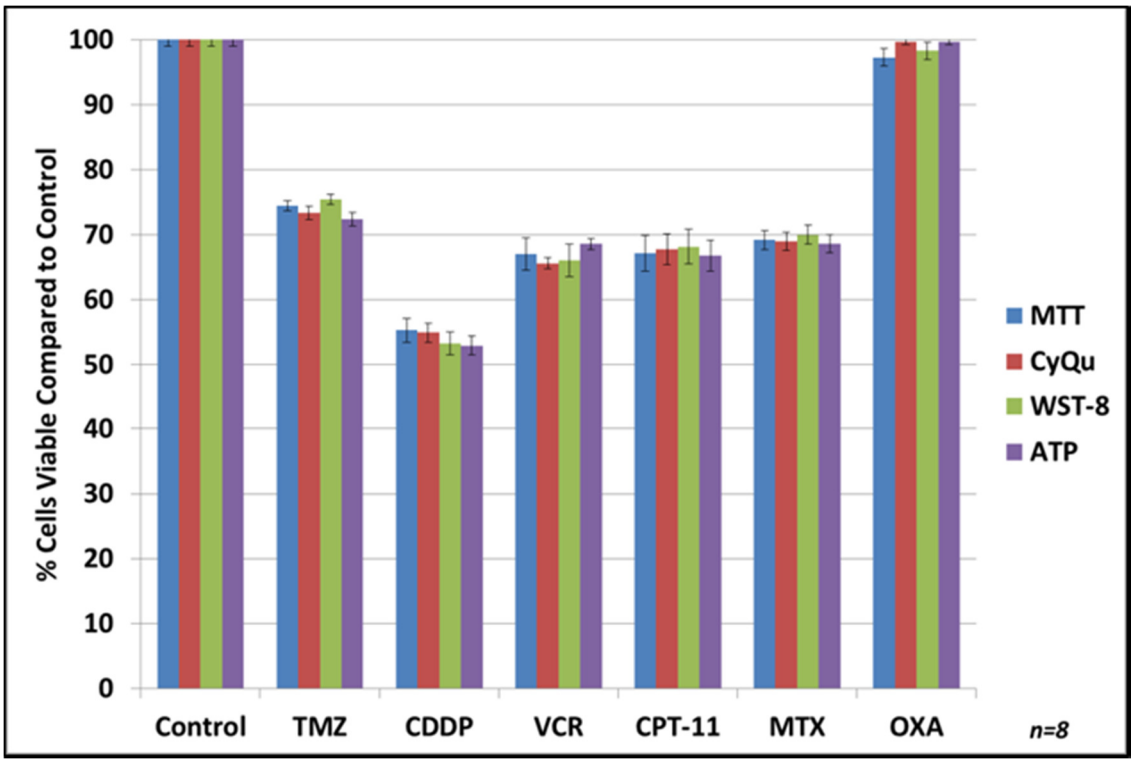

Figure 2. MTT vs. CyQUANT vs. WST-8 vs. ATP.

Using a GBM primary cell line to compare MTT assay, CyQUANT, WST-8 and ATP luciferase assay. Each assay was plated with $1x10^4$ cells per well. All assays were treated with one-hour pulse of chemotherapeutic agents then analyzed 24 hours later. *P*-value was greater than 0.5 when comparing the assays.

Isolation of CSLCs from the tumors was accomplished by either magnetic bead sorting of the CD133+ population or by using the Hydrodynamic Focusing Bioreactor (HFB). To determine if these methods produced the same results, different isolation methods were compared and found that the response to the drugs was not statistically significant (Figure 3). However, it was also determined that in order to obtain the necessary number of sub-confluent plates of cells to be used in a magnetic bead sort of CD133+ cells useful to perform a chemosensitivity assay it would take around two months of tissue culture vs. the seven days process when using the HFB (45).

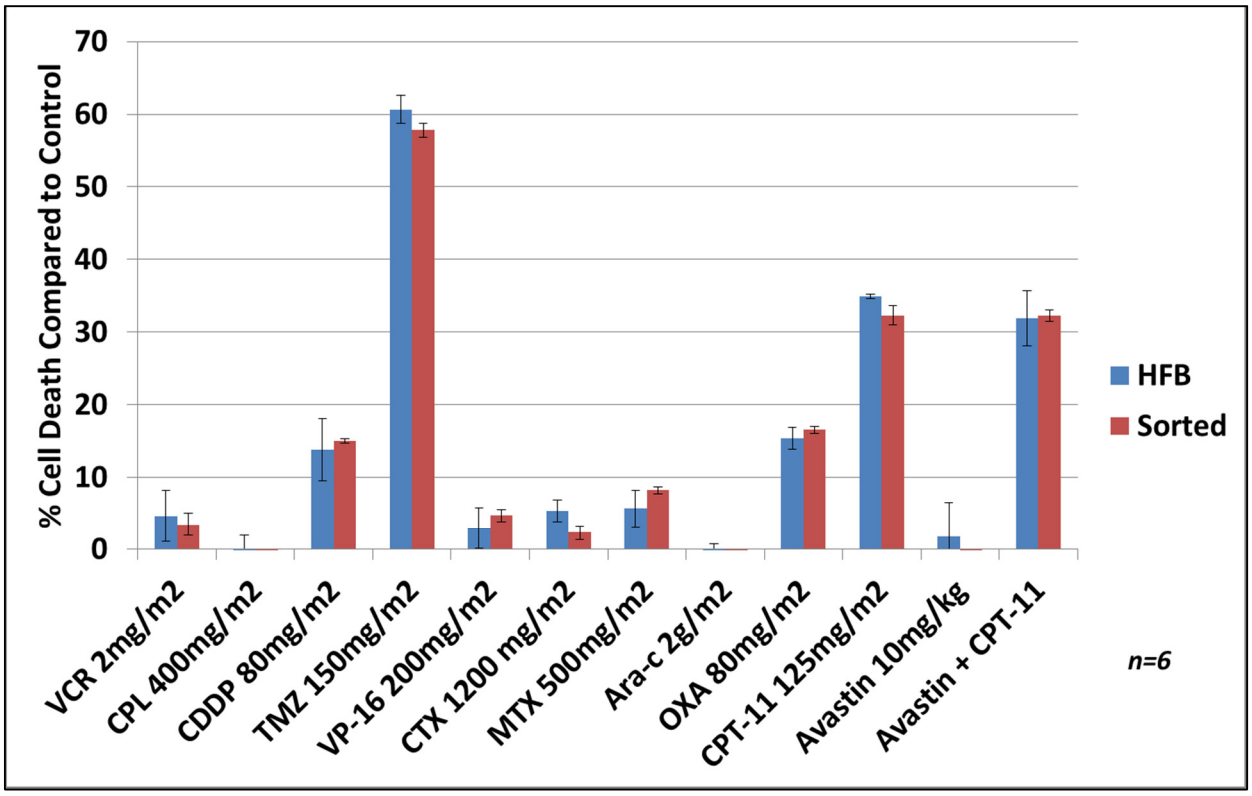

Figure 3. Drug Response to: HFB vs. MACSorted CD133 Isolation.

Same GBM cell line was used to compare the CD133 population of cells isolated by HFB vs. MACSorted CD133 Microbeads kit. Each assay was plated with $1x10^4$ cells per well. All assays were treated with one hour pulse of chemotherapeutic agents then analyzed 24 hours later with MTT. *P-value* was greater than 0.5 when comparing the assays.

After bulk of tumor cells and CSLCs of each primary patient cell line were tested to determine the response to the various drugs by MTT assay, the samples were analyzed by calculating the percentage of cellular viability against the control using the following formula:

$$(Average\ of\ drug\ tested / Control\ Average) * 100 = \% Viable\ Cells\ compared\ to\ Control$$

Percent of non-viable cells were calculated using the following formula: $100 - \% Viable\ Cells = \% Cell\ Kill$. Drug response was reported by assigning samples in which it was found that there was between 60-100% Cell Kill to a responsive category; followed by 30-59.9% Cell Kill to be intermediate responsive; and 29.9-0% to be non-responsive.

Standard deviation was calculated off of the mean absorbance of the MTT assay for each treatment and multiplied by 100 to get the percent standard deviation. If this number was greater then 5% one value would be removed using the student t-test. If less than 5% standard deviation was still not achieved then whole test would be run again.

Disease outcomes were correlated to the determination of chemosensitivity to the specific chemotherapy drug that was administered to the patient to determine clinical correlation with the *in vitro* chemosensitivity assay.

Chemotherapy Sensitivity Assay Validation

This assay has been recently validated in compliance with CLIA regulations (42CFR493.1291 (3)). Accuracy and precision were assayed by a minimum of three (3) repeats of the chemotherapy sensitivity assay, with three (3) established cell lines with unique chemotherapeutic response profiles covering the spectrum of the detectable range of <10% to 100% cell viability.

Quantitative agreement, with less than 30% standard deviation, of cell viability for cell line HEK293, less than 16% standard deviation for cell line SW480, and less than 18% standard deviation cell viability for cell line DU145 were the intended goals. These standard deviations were determined by assaying growth control plates for the established cell lines which were used to ascertain their base mitochondrial activity in relation to cell viability.

Growth control plates were established by plating 10,000 cells/ml of each cell line in a series of wells. These plates followed the same incubation periods and were treated the same as a regular assay as outlined in the chemotherapy sensitivity assay, except they were not treated with any chemotherapeutics. Absorbance readings were taken and from those, percent cell viability was calculated. The difference in the range of cell viability determined the acceptable percent standard deviation for that cell line.

Repeatability was measured by the performance of two runs of the assay for each chemotherapeutic (dosage and infusion rate) by the same person. Agreement between these two runs is expected to be less than the acceptable standard deviation for that cell line determined by the growth control plate.

Reproducibility was measured by the performance of the test being analyzed on discontinuous days by a different person and contributed to the overall accuracy of the test.

Within-run repeatability was assessed by the four (4) repeats per chemotherapeutic per assay that are standard to the chemotherapy sensitivity assay. The standard deviation of these four repeats shall be no greater than 5%. One outlier per the four repeats per chemotherapeutic can be thrown out per standard operating procedure for the chemotherapy sensitivity assay and still achieve statistical significance.

Repeatability: 99.5% of all repeats matched within the acceptable standard deviation. Reproducibility: 95.5% of all repeats matched within the acceptable standard deviation. Within-Run Repeatability: 99.7% of all repeats per chemotherapeutic per run matched within a 5% standard deviation.

Analytical sensitivity was tested by treating 10,000 cells/ml with 100µl of 100% ethanol. Ethanol, used as a positive control in the chemotherapy sensitivity assay, will kill the majority of the living cells. The resultant absorbance readings showed that cell viability ranged from 1.2% to 11.2% across varying cell lines showing that chemotherapy sensitivity assay can detect living cells in the range of 120 – 1,120 cells/ml. Thus a range of <10% cell viability is the minimum limit of the chemotherapy sensitivity assay's sensitivity to be reported on diagnostic tests.

Analytical specificity was tested by attempting chemotherapy sensitivity assay on non-neoplastic cells. The percentage of normal stem cells that grew or survived during the initial growth period prior to chemotherapy analysis was too low to perform the chemotherapy sensitivity assay showing that only neoplastic cells can survive the culture growth period in testable numbers.

Robustness of this lab developed test was assessed by observing the culture growth of CNS tumor tissue samples stored in varying media and conditions. This condition was successful in producing enough cells for analysis: RPMI-1640 + 10% FBS + 2% P/S stored at room temperature. These conditions were deemed unsuccessful for chemotherapy sensitivity assay analysis: RPMI-1640 + 10% FBS + 2% P/S stored at 4ºC for up to 2 hrs, Saline stored at room temperature, and Ethanol stored at room temperature.

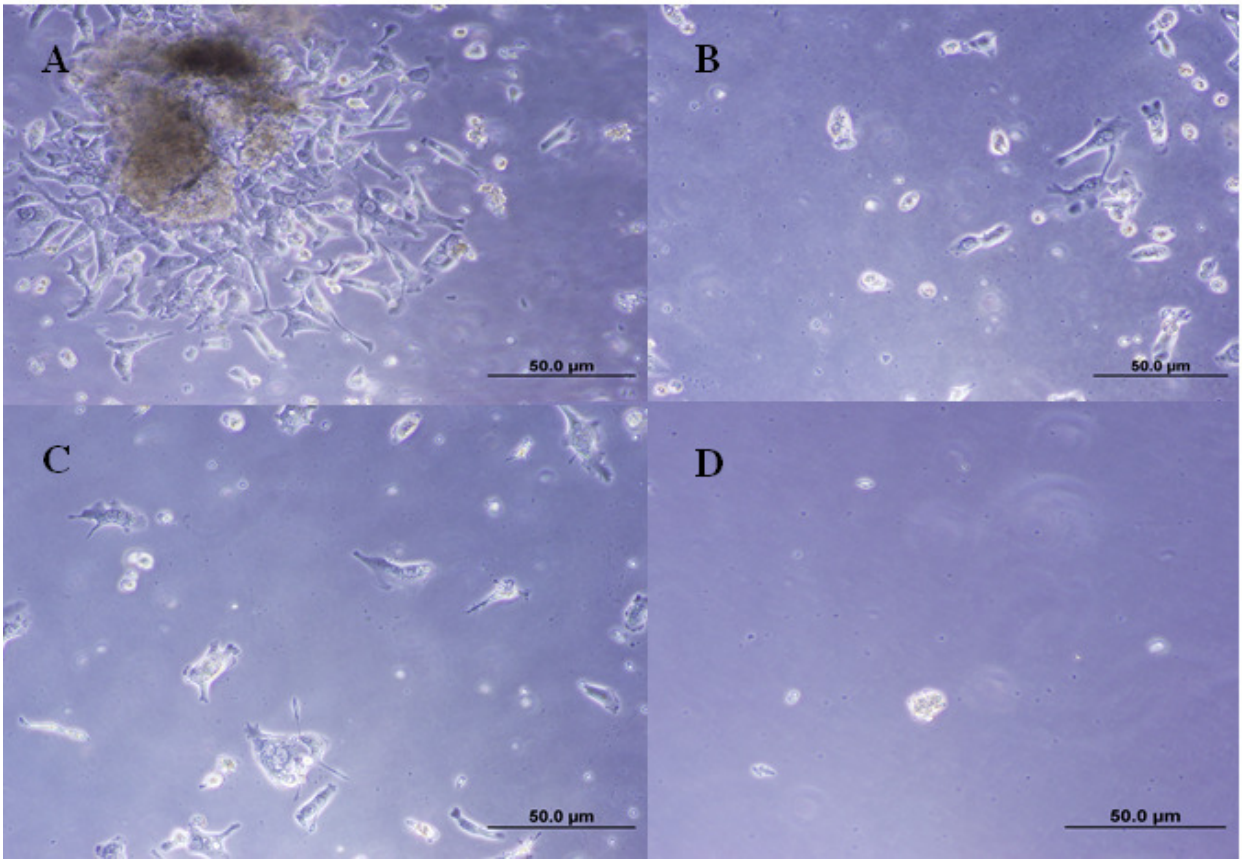

Figure 4. Robustness Testing on Primary Tumor Tissue.

A) Media: RPMI + 10% FBS + 2% P/S Storage condition: Room temp. Growth period: expected growth after 7-day incubation. B) Media: RPMI + 10% FBS + 2% P/S Storage condition: 4ºC for 2 hrs. Growth period: no growth after 7-day incubation. C) Media: Saline Storage condition: Room temp for 2 hrs. Growth period: slow growth after 7-day incubation. D) Media: Ethanol Storage condition: Room temp for 2 hrs. Growth period: slow to no growth after 7-day incubation.

Reportable range was confirmed by testing cell lines ranging from <10% viability to 100% viability and unique chemotherapeutic response profiles were generated for each cell line. Cell lines successfully repeated among these ranges.

CONCLUSIONS

The drug response assay tested for both the sensitivity to standard-of-care drugs on the overall tumor (Bulk) and the CSLC population. By challenging these cells with the different chemotherapeutic agents the test was able to determine ineffective drugs that could be excluded from the panel of drugs used by the treating physician.

This study investigated 4 cases of CNS malignant tumors, two Ependymoma and two Glioblastoma. When these cases were treated with standard-of-care chemotherapy drugs the tumors should have responded in a similar fashion, however, the data accrued in this study shows that patients had better outcomes when treated with effective chemotherapies against CSLC. It is important to note that response of CSLCs to the treating chemotherapies indicated the patient disease outcome in both cases. While many other CNS cases have been studied with this method, the data relative to these cases is being analyzed and it will be the subject matter of a future publication.

CHAPTER 2: CASE STUDY OF EPENDYMOMA

This manuscript is a revised version of Chemo-Predictive Assay for Targeting Cancer Stem-Like Cells in Patients Affected by Brain Tumors from *PlosOne* (2014).

Mathis, S. E., Alberico, A., Nande, R., Neto, W., Lawrence, L., McCallister, D. R., Denvir, J., Kimmey, G. A., Mogul, M., Oakley, G., 3rd, Denning, K. L., Dougherty, T., Valluri, J. V., and Claudio, P. P. (2014) Chemo-predictive assay for targeting cancer stem-like cells in patients affected by brain tumors. *PloS one* **9**, e105710

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ABSTRACT

Administration of ineffective anticancer therapy is associated with unnecessary toxicity and development of resistant clones. Cancer stem-like cells (CSLCs) resist chemotherapy, thereby causing relapse of the disease. Thus, development of a test that identifies the most effective chemotherapy management offers great promise for individualized anticancer treatments. This study was designed to investigate an *ex vivo* chemotherapy sensitivity assay, which measures the sensitivity of CSLCs as well as the bulk of tumor cells to a variety of chemotherapy agents. Two patients, a 21-year old male (patient 1) and a 5-month female (patient 2), affected by anaplastic WHO grade-III Ependymoma were screened using the chemotherapy sensitivity assay. Patient 1 was found sensitive to the combination of Irinotecan and Bevacizumab, which resulted in a prolonged disease progression free period of 18 months. Following recurrence, the combination of various chemotherapy drugs was tested again with the chemotherapy sensitivity assay. This study found that benzyl isothiocyanate (BITC) greatly increased the chemosensitivity of the Ependymoma cells to the combination of Irinotecan and Bevacizumab. After patient 1 was treated for two months with Irinotecan, Bevacizumab and supplements of cruciferous vegetable extracts containing BITC, 50% tumoral regression was achieved in comparison with pre- chemotherapy sensitivity assay scan as evidenced by MRI. Patient 2 was found resistant to all treatments tested and following 6 cycles of Vincristine, Carboplatin, Cyclophosphamide, Etoposide, and Cisplatin in various combinations, the tumor of this patient rapidly progressed and proton beam therapy was recommended. As expected animal studies conducted with patient derived xenografts treated with chemotherapy sensitivity assay screened drugs recapitulated the clinical observation. This assay demonstrates that patients with the same histological stage and grade of cancer may vary considerably in their clinical response,

suggesting that chemotherapy sensitivity assay testing which measures the sensitivity of CSLCs as well as the bulk of tumor cells to a variety of chemotherapy agents could lead to more effective and personalized anticancer treatments in the future.

INTRODUCTION

Although Ependymomas are the third most common type of brain tumor in children (following Astrocytoma and Medulloblastoma), they are relatively rare, with approximately 200 cases diagnosed in the US each year (59,60). They account for 60% of all intramedullary tumors and 50% arise in the *filum terminale* (61).

The treatment of Ependymoma can be challenging. The initial standard treatment for Ependymoma is surgery often followed by radiation therapy and chemotherapy. Although chemotherapy has been used extensively in children with Ependymoma, there is little clinical evidence that chemotherapy improves survival of children with this type of tumor. Chemotherapy is often reserved for patients with residual tumor after surgery and for children younger than 3 years of age in an attempt to delay radiation therapy (62).

It is not entirely clear why there is not an improved survival with chemotherapy, but it is known that resistance to a variety of commonly used chemotherapeutic agents is common in Ependymoma (63). Therefore investigation and development of novel strategies and integrated therapies are required to find more effective treatments for this type of tumor.

Patients with the same stage and grade of cancer may vary considerably in their clinical response and toleration of chemotherapy. Ineffective anticancer therapy can result in unnecessary toxicity and the development of resistant clones. The surviving cancer cells are often more resistant to therapy. Many attempts have been made over the years to develop an *ex-vivo* anti-cancer test that could help discern the best treatment options for each individual patient while minimizing toxicity.

Animal xenograft models have shown that only a subset of cancer cells within each tumor is capable of initiating tumor growth. This capability has been shown in several types of human

cancers, to include Ependymoma (64). This pool of cancer cells is operationally defined as the ‘‘Cancer Stem-Like Cell’’ (CSLC) subset. According to the ‘‘cancer stem-like cell’’ theory, tumors are a complex, growing population of abnormal cells originating from a minority of CSLCs. These cells maintain stem-like characteristics in that they proliferate very slowly and have an inherent capacity to self-renew and differentiate into phenotypically heterogeneous, aberrant progeny (45,65-67). Unlike the bulk of tumor cells, CSLCs resist chemotherapy and radiation therapy and are responsible for tumor relapse and metastasis (66,67).

Some Ependymoma express various markers of stemness, including CD133. In addition, relapsed tumors exhibit a gene expression signature constituted by up-regulated genes involved in the kinetochore (ASPM, KIF11) or in neural development (CD133, Wnt and Notch pathways) (68).

Targeting CSLCs in addition to the bulk of other cancer cells within a tumor is a new paradigm in cancer treatment. This recent study shows that a Hydrodynamic Focusing Bioreactor (HFB) (Celdyne, Houston TX) selectively enriches CSLCs from cancer cell lines that can be used in a chemosensitivity assay (45). Further, using this strategy to optimize the enrichment of CSLCs from tumor biopsies has become essential to the development of the chemotherapy sensitivity assay, which measures the response of CSLCs and the bulk of tumor cells to chemotherapy to determine the most effective combination of anticancer drugs for malignant tumors of the nervous system.

In this study it is reported for the first time, an investigation using the chemotherapy sensitivity assay to measure the sensitivity and resistance of CSLCs and bulk of tumor cells. It was performed on two biopsies cultured from human Ependymoma. These tumors were challenged with several chemotherapy agents which were also correlated to the response of

animal xenografts treated with the predicted drugs and to the clinical response of the treated patients.

MATERIALS AND METHODS

Patients

Case 1 is a 21-year-old male patient diagnosed with intradural, intramedullary, and extramedullary anaplastic diffuse spinal Ependymoma, WHO grade III. Case 2 is a 5-month old female patient diagnosed with anaplastic WHO grade III Ependymoma. The chemotherapy sensitivity assay was performed after obtaining patient's or guardian's written informed consent in accordance with the ethical standards of the Helsinki Declaration (1964, amended most recently in 2008) of the World Medical Association. Any information, including illustrations, has been anonymized. Marshall University Institutional Review Board (IRB) has approved this research under the protocol #326290. Participants or guardians of participant (in case of a child participant) provided their written consent on an IRB approved informed consent form to participate in this study after being educated about the research protocol. Ethics committees/ IRB at Marshall University approved this consent procedure. For children participants to the study, written informed consent was obtained from the next of kin, caretakers, or guardians on behalf of the minors/children enrolled in the study.

Single Cell Suspension and Primary Cell Culture

Single-cell suspensions from the Ependymoma biopsies were prepared using the gentleMACS Dissociator (Miltenyi, Auburn, CA), and C Tubes using a standardized, semi-automated protocol based on a combination of mechanical tissue disruption and incubation with a 50% solution of a 0.025% Trypsin and Accutase (Innovative Cell Technologies, San Diego, CA). Cells were serially plated in 24-well, 12-well, 6-well, 10-cm treated dishes and cultured to subconfluence in RPMI-1640 medium supplemented with 5% irradiated, heat inactivated,

defined fetal bovine serum (Thermofisher/Hyclone), and 50 U of penicillin and 5 mg of streptomycin/mL of medium (Thermofisher/Mediatech).

Reagents

Benzyl isothiocyanate (BITC) was purchased from Sigma Chemical Co. (St. Louis, MO). Bevacizumab (Avastin), Cisplatin, Oxaliplatin, Arabinoside-C, VP-16, Irinotecan (Camptosar, CPT-11), Busulfan, Methotrexate, were acquired as clinical grade chemotherapy agents from Edwards Cancer Center and St. Mary's Hospital.

Three-Dimensional Bioreactor CSCs Culture

A Hydrodynamic Focusing Bioreactor (HFB) (Celdyne, Houston TX) was used as previously described to selectively proliferate CD133 (+) cancer stem-like cells (45). Culture media, oxygenation, speed, temperature and CO2 were kept constant for ten days. Cells were counted and $1x10^6$ cells were placed in the rotating vessel set at 25 rpm with airflow set at 20%. Cells were then removed and counted again using trypan blue exclusion to determine cellular viability and cell number and plated in 96 wells for chemosensitivity testing. The cells were also incubated with florescent antibodies for phenotypic characterization (45).

Cell Sorting

Up to $1x10^6$ cells were sorted by a magnetic-activated cell sorting (MACS) system, which consists of magnetic beads conjugated to an antibody against CD133 (Miltenyi, Auburn, CA). In brief, cells were harvested using 0.25% trypsin, pelleted and labeled with CD133/1 biotin and CD133/2-PE. Cells were washed and labeled with anti-biotin magnetic beads, and

then passed through a magnetic column where CD133 (+) cells were retained, while unlabeled cells passed through the column. The CD133 (+) retained cells were eluted from the columns after removal from the magnet. Positive and negative cells were then analyzed by FACS for purity.

Flow Cytometry Studies

Cells were analyzed by the antigenic criteria using anti-CD34 (Milteny Biotech, Auburn, CA), -CD38 (Milteny Biotech, Auburn, CA), -CD44 (BD Bioscience, Sparks, MD), -CD117 (Milteny Biotech, Auburn, CA), -CD133/2 (prominin1) (Milteny Biotech, Auburn, CA), -Oct3/4 (BD Bioscience, Sparks, MD), and –Nanog (BD Bioscience, Sparks, MD). Briefly, cells were detached using 0.02% EDTA in PBS and pelleted (10 min at 1,000 rpm), washed in 0.1% BSA in 1X PBS at 4°C and incubated in a solution of 1 mg antibody +9 mL 0.1% BSA in 1X PBS. Cells were washed in the same solution once and were analyzed using a C6 Accuri flow cytometer (BD Biosciences, San Jose, CA).

Chemotherapy Sensitivity Assay

Sensitivity to chemotherapy was assessed using a viability assay (WST8) on $1x10^3$ cells plated in five replicas into 96-well plates. Briefly, equal number of bulk of tumor cells grown in monolayer and CSLCs grown in the bioreactor were counted and seeded separately in 96-well dishes and incubated at 37°C for 24 hours. The cells were then challenged with a one-hour pulse of a panel of anticancer drugs as chosen by the oncologist to mimic the average clinical chemotherapy infusion schedule.

To study the effect of BITC on chemosensitization of cancer cells to chemotherapy drugs, the cells were treated with an hour pulse 5–30 mM BITC followed by an hour of the various anticancer drugs. Each anticancer drug was tested in a range of doses including the clinically relevant dose.

A WST8 assay was performed 48 hours following chemotherapy treatment to assess cell viability as previously described (69). A dose response chart was developed in which samples were scored as responsive (0–30% cell survival), intermediate (30–60% cell survival), and non-responsive (60–100% cell survival).

Limiting Dilution Tumorigenic Assay in Immune Deficient Mice

A range of $1x10^2$; $1x10^3$; $1x10^4$; and $1x10^5$ Ependymoma cells from Patient 1 were injected subcutaneously in five athymic immunodeficient nude nu/nu mice per group. Briefly, an equal number of parental bulk of tumor cells grown in 2D monolayer, CD133 (+) three-dimensionally grown in the hydrofocusing bioreactor, and CD133 (+) MACSorted CSLCs were injected with 100 mL of matrigel in the flank of NOD-Scid mice and compared to the growth of CD133 negative cells for three months.

Animal Study

All animal studies have been conducted following approval from the Marshall University IACUC, protocol #373017. The effects of chemotherapies screened *in vitro* by the chemotherapy sensitivity assay were tested on human tumor biopsies that were xenografted in the flank of a NOD-Scid mouse model. $1x10^6$ Ependymoma cells were mixed to 100 uL of matrigel (BD Biosciences, San Jose, CA) injected subcutaneously in the flank of ten athymic, NOD.Cg- Prkdc

Scid ll2rgtm1wjl/SzJ immunodeficient mice (NOD-Scid)/group and were grown for 10 weeks or until 100 mm^3. Mice were randomized in different treatment and control groups and chemotherapy was administered by intraperitoneal (i.p.) injections in 200 uL as follows in a period of four weeks: 1) Group #1, Control group with primary tumor cells injected into flank and receiving i.p. sterile saline injections. Group #2, Experimental group injected i.p. with the least effective chemotherapy as determined by the *in vitro* chemotherapy sensitivity assay. Group #3, Experimental group injected i.p. with the most effective chemotherapy as determined by the *in vitro* chemotherapy sensitivity assay. Group #4, Experimental group injected i.p. with the second most effective chemotherapy as determined by the *in vitro* chemotherapy sensitivity assay. Group #5, Experimental group injected i.p. with the most effective combinatorial chemotherapy as determined by the *in vitro* chemotherapy sensitivity assay.

Chemotherapy doses for the animal study were calculated using a body surface area (BSA) normalization method (70) from the clinical dose and verified according to doses previously determined by a literature search.

Animals were euthanized following the current guidelines established by the latest Report of the AVMA Panel on Euthanasia using CO2 inhalation and asphyxiation followed by cervical dislocation.

Statistical Analysis

Statistical analysis was performed using the IBM SPSS statistical software. The results for each variant in the different experimental designs represent an average of three different experiments. The data of five measurements were averaged; the coefficient of variation among these values never exceeded 10%. Mean values and standard errors were calculated for each

point from the pooled normalized to control data. Statistical analysis of the significance of the results was performed with a one-way ANOVA. P values of less than 0.05 were considered statistically significant.

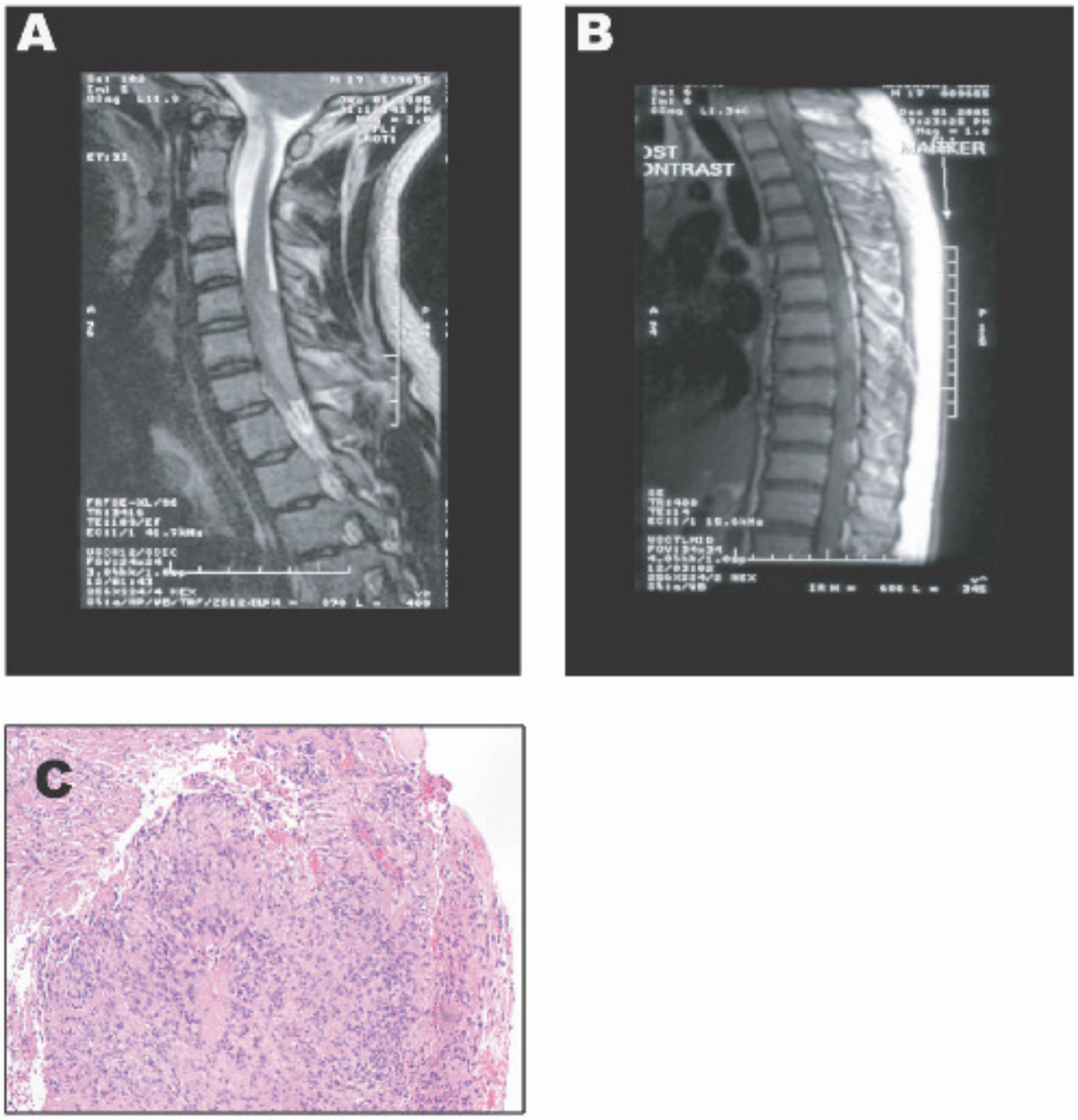


Figure 5. MRI Images and H&E Staining of the Anaplastic Ependymoma Case 1 at Presentation.

A) Magnetic Resonance Imaging (MRI) of the cervical spine showing the presence of an enhancing mass, which extends from mid C5 to inferior C7 (4.5 in length x 1.0 x 2.0 in cephalocaudal and anteroposterior dimension) and causing cord compression.
B) MRI of the thoracic spine showing an enhancing lesion at T2–3 (1.5 in length x 0.6 x 0.6 cm in anteroposterior and transverse dimension) with several other smaller nodular masses, best seen on the T2 weighted sequence, which extended throughout the thoracic level to T11.
C) Hematoxylin and Eosin staining of a tumor section showing an overall predominant dense cellular component, with primitive nuclear features, mitotic activity, necrosis and vascular proliferation. The presence of well formed, obvious perivascular pseudorosettes (with vasocentric pattern, perivascular nuclear-free zones, and classic thin glial processes radiating to/from the vessel wall) were found supportive of the diagnosis of intradural, extramedullary anaplastic diffuse spinal Ependymoma, WHO grade III. doi:10.1371/journal.pone.0105710.g001

RESULTS

Ependymoma Patient 1

A physically active 17-year-old male presented in October 2005 with paresthesia in his feet and a rather severe perceptive loss. This became progressively worse in December 2005 going up his legs with rather severe numbness in the right leg and pain in his left leg, from the mid-thigh down to the mid-calf medially. On examination he had no focal weakness throughout his upper and lower extremities. He had hypoalgesia with partial sensory level in the upper thoracic spine down. He also had severe proprioception loss in his feet and toes. Magnetic resonance imaging (MRI) of the cervical spine showed the presence of an abnormal enhancing mass, which extended from mid C5 to inferior C7 (4.5 in length x 1.0 x 2.0 in cephalocaudal and anteroposterior dimension) that caused cord compression (Figure 5A). MRI of the thoracic spine showed an enhancing lesion at T2–3 (1.5 in length x 0.6 x 0.6 cm in anteroposterior and transverse dimension) with several other smaller nodular masses, best seen on the T2 weighted sequence, which extended throughout the thoracic level to T11 (Figure 5B).

The patient received a laminectomy in December 2005 at C5, C6, and C7 with partial resection of the tumor under microscope using microsurgical techniques. Following surgery, the patient was treated with radiation and temozolomide. Morphological analysis of the histology sections stained with Hematoxylin & Eosin showed an overall predominant dense cellular component, with primitive and pleomorphic nuclei, increased mitotic rate and apoptosis, and foci with microvascular proliferation. The presence of well formed, obvious perivascular pseudorosettes (with vasocentric pattern, perivascular nuclear-free zones, and classic thin glial processes radiating to/from the vessel wall) were found supporting the diagnosis of anaplastic diffuse spinal Ependymoma, WHO grade III.

Figure 5C shows the hematoxylin and eosin staining of a tumor section at diagnosis in 2005. Sections of the tumor were evaluated by immunoperoxidase techniques with appropriate staining control sections. The tumor showed positive staining with antibodies to neuron specific enolase, vimentin, S-100, and GFAP. Weak staining occurred with the antibodies against actin. Focal staining occurred with antibodies to epithelial membrane antigen, cytokeratin AE1/AE3, and synaptophysin. The tumor was negative for leukocyte common antigen, desmin, and myogenin. In addition, a section stained with PAS showed a focal PAS-positive fibrillar material. Sections and tumor block were also sent to the Biopathology Center (BPC) of the Children's Oncology Group (COG) where two neuropathologists independently reviewed the case and confirmed the diagnosis of Anaplastic Ependymoma, WHO grade III.

Following recurrence and progression, the patient received complex chemotherapy regimen in January 2006 and March 2006 with Cyclophosphamide, Thalidomide, Celecoxib followed by Etoposide, Thalidomide and Celecoxib. Chemotherapy treatment was concluded in September of 2006, but in August of 2007 patient had tumor regrowth at T7–T8 for which he underwent robotic radiosurgery treatment. The patient had another debulking surgery in April of 2008, but later in December of 2008 he had progressive numbness in his legs along with back pain with MRI showing recurrence in the surgical area (Figure 6A) as well as the lumbar spine. He was then treated again with Temozolomide, but had no response to treatment.

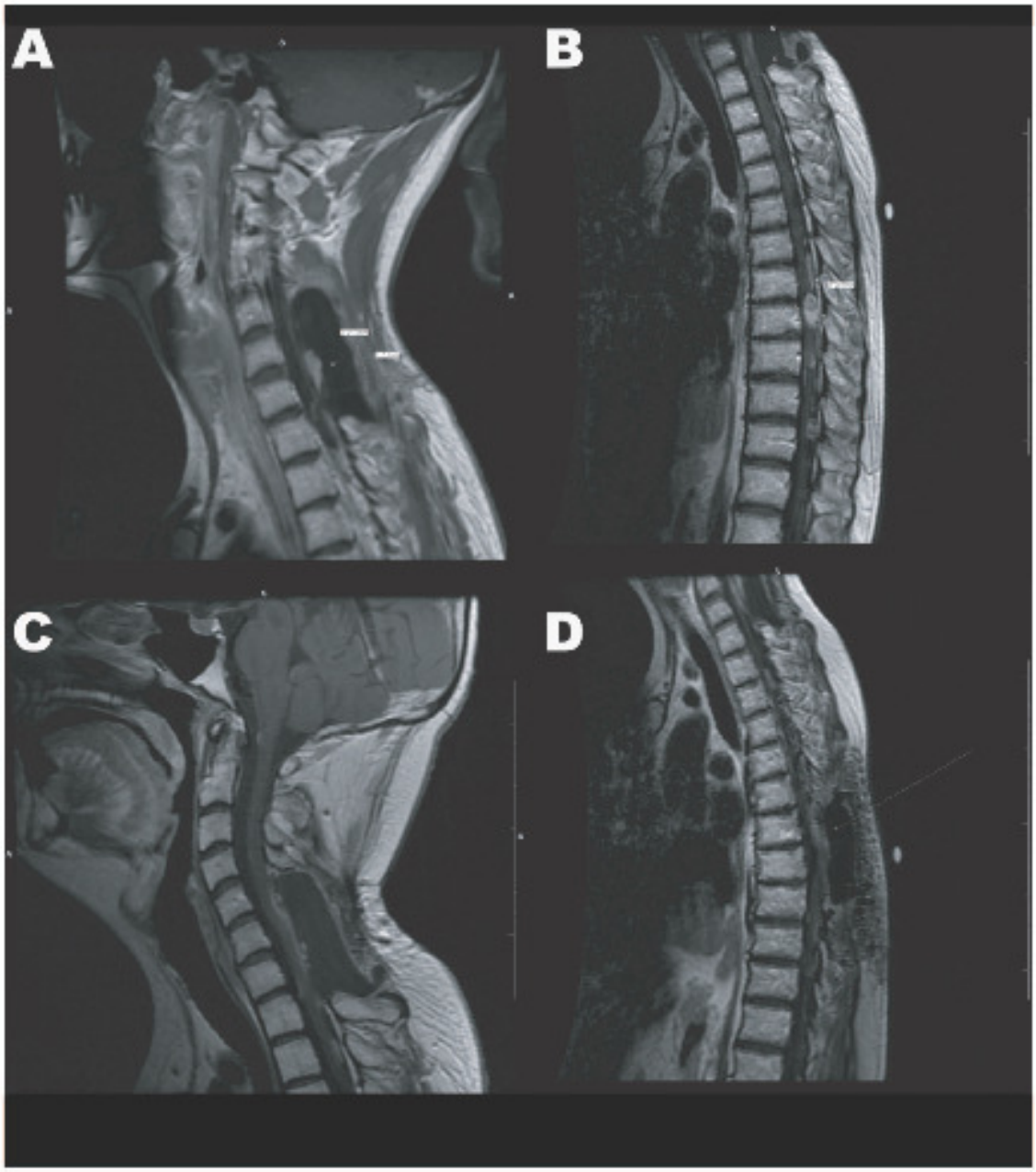


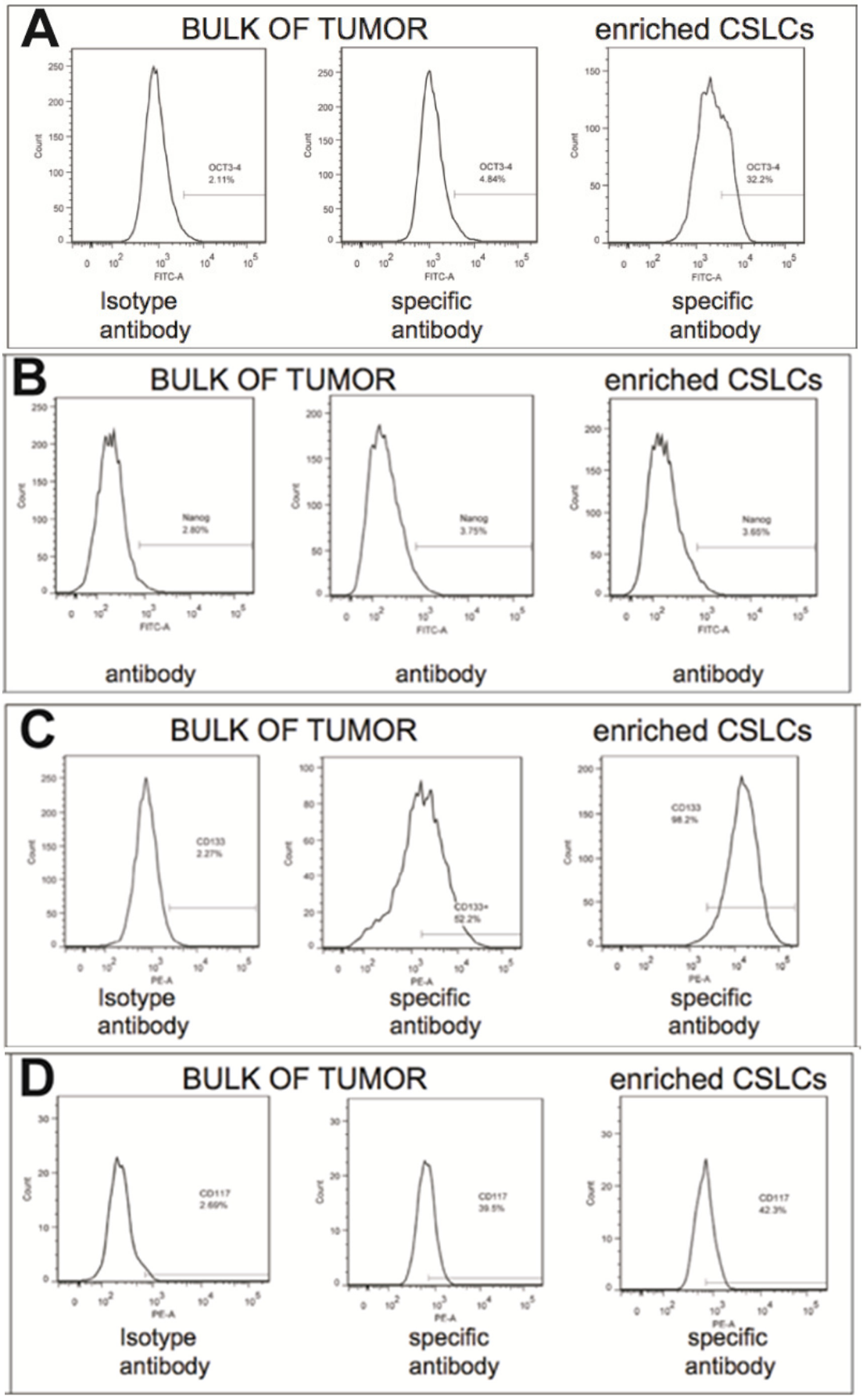
Figure 6. MRI Images of Cervical and Thoracic Spine of Ependymoma Case 1.

A) 2009 MRI of the cervical spine showing recurrence in the surgical area. B) 2009 MRI of the thoracic spine showing progression of the main lesion measuring 23.9 mm, and the appearance of several other smaller lesions. C and D) 2010 MRI of the cervical and thoracic spine showing tumor regression following a treatment with Irinotecan and Bevacizumab. doi:10.1371/journal.pone.0105710.g002

In March 2009 because of progression of the disease he had a thoracic laminectomy and resection of the intradural intramedullary tumor. He had severe spinal compression and began having weakness in his legs. Due to further recurrence, the patient then had another debulking surgery in July of 2009. He also received Oxaliplatin and Etoposide treatment in July and August 2009, but the tumor progressed even more (Figure 6B). Appropriate informed consent was signed and at the time of the debulking surgery of July 2009, a sterile biopsy was taken to assess the sensitivity of the tumor cells (bulk of tumor and CSLCs) toward standard-of-care chemotherapy drugs using the chemotherapy sensitivity assay. The biopsy was placed in RPMI-1640 sterile media and tissue was dissociated in the laboratory into a single-cell suspension with the use of a GentleMACS tissue dissociator (Miltenyi, Aubourn, CA). The single-cell Ependymoma suspension was plated in RPMI-1640 in the presence of 5% irradiated, heat inactivated, defined fetal bovine serum, streptomycin and penicillin and cells were cultured as a monolayer for 15 days. Cells were immunophenotyped by flow cytometry using antibodies against CD34, CD38, CD44, CD117, CD133, OCT3/4, and Nanog.

The Ependymoma cells were found positive to OCT3/4 (2.73%), Nanog (0.95%), CD133 (49.93%), CD117 (36.81%), and CD44 (20.39%) when compared to an isotype control antibody (Figure 7 A-E). A double staining of CD34 and CD38 showed the presence of 1.88% of the cells CD34+/CD38+, and 78.4% CD34+/CD38- cells (Figure 7F). To expand the CSLC population of CD133+ cells from the Ependymoma primary culture, the Ependymoma cells were cultured as previously described (45). $1x10^6$ of the Ependymoma cells from a monolayer primary culture were grown for ten days using Hydrodynamic Focusing Bioreactor (HFB) (Celdyne, Houston, TX) (45). The Ependymoma cells cultured in the bioreactor formed cell clusters (Figure 8A)

which were expanded 14.7 fold (Table 1) and appeared to be 95.93% CD133 positive after 10 days of culture in the bioreactor (Figure 7 C, enriched CSLCs).


A
BULK OF TUMOR
enriched CSLCs
OCT3-4
2.11%
OCT3-4
4.84%
OCT3-4
32.2%
Count
FITC-A
Isotype antibody
specific antibody
specific antibody
B
BULK OF TUMOR
enriched CSLCs
Nanog
2.80%
Nanog
3.75%
Nanog
3.65%
antibody
antibody
antibody
C
BULK OF TUMOR
enriched CSLCs
CD133
2.27%
CD133+
52.2%
CD133
98.2%
PE-A
Isotype antibody
specific antibody
specific antibody
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CD117
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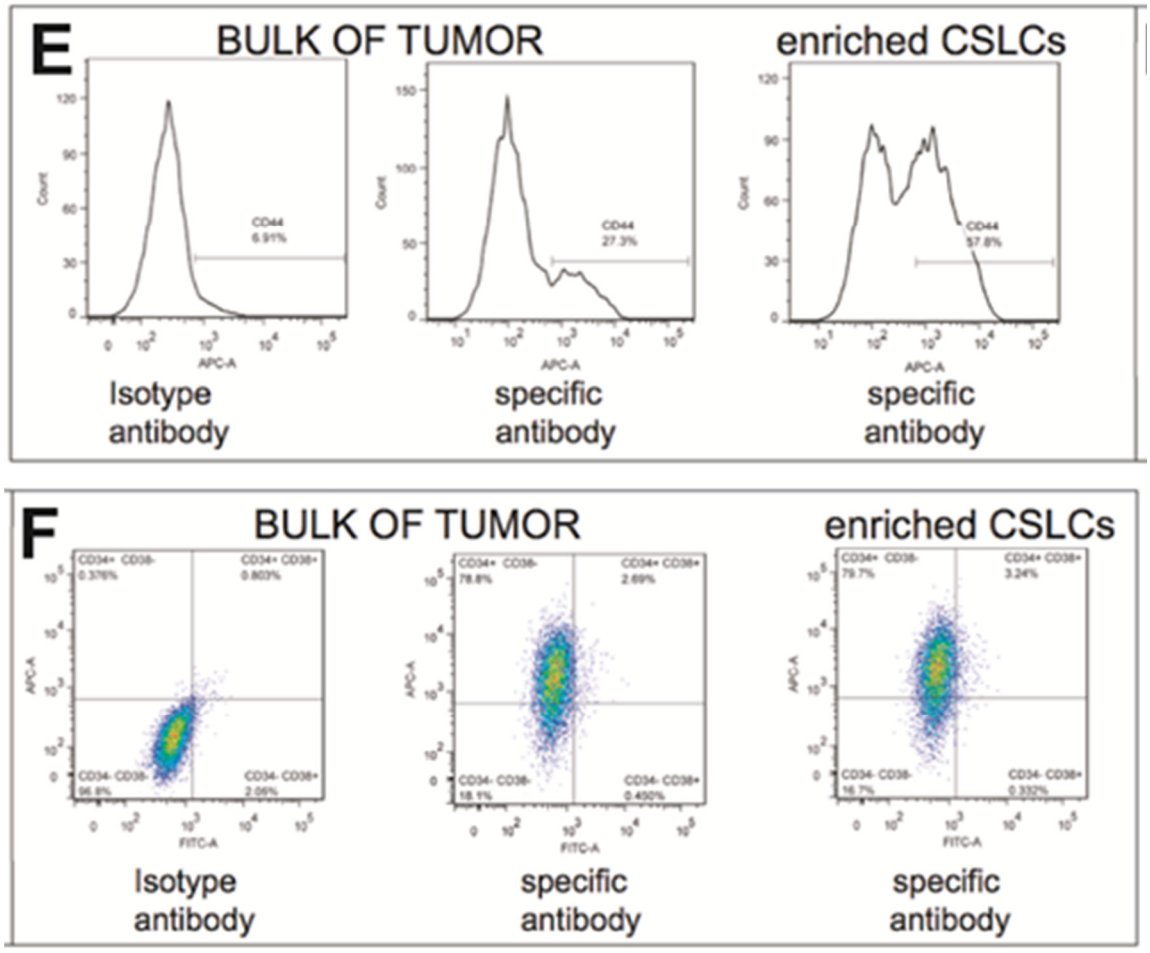


Figure 7. Characterization of the Primary Ependymoma Cell Culture and of the Enriched CSLCs of Case 1.

A-F) Immunophenotype conducted using: A) OCT3/4 antibody; Left panel: isotype antibody (bulk of tumor cells); Center panel: specific antibody (bulk of tumor cells); Right panel: specific antibody (enriched CSLCs). B) Nanog antibody; Left panel: isotype antibody (bulk of tumor cells); Center panel: specific antibody (bulk of tumor cells); Right panel: specific antibody (enriched CSLCs). C) CD133 antibody; Left panel: isotype antibody (bulk of tumor cells); Center panel: specific antibody (bulk of tumor cells); Right panel: specific antibody (enriched CSLCs). D) CD117 antibody; Left panel: isotype antibody (bulk of tumor cells); Center panel: specific antibody (bulk of tumor cells); Right panel: specific antibody (enriched CSLCs). E) CD44 antibody; Left panel: isotype antibody (bulk of tumor cells); Center panel: specific antibody (bulk of tumor cells); Right panel: specific antibody (enriched CSLCs). F) Double labeling with CD34 and CD38 antibodies; Panel on left: isotype antibody (bulk of tumor cells); Center panel: specific antibody (bulk of tumor cells); Panel on right: specific antibody (enriched CSLCs).

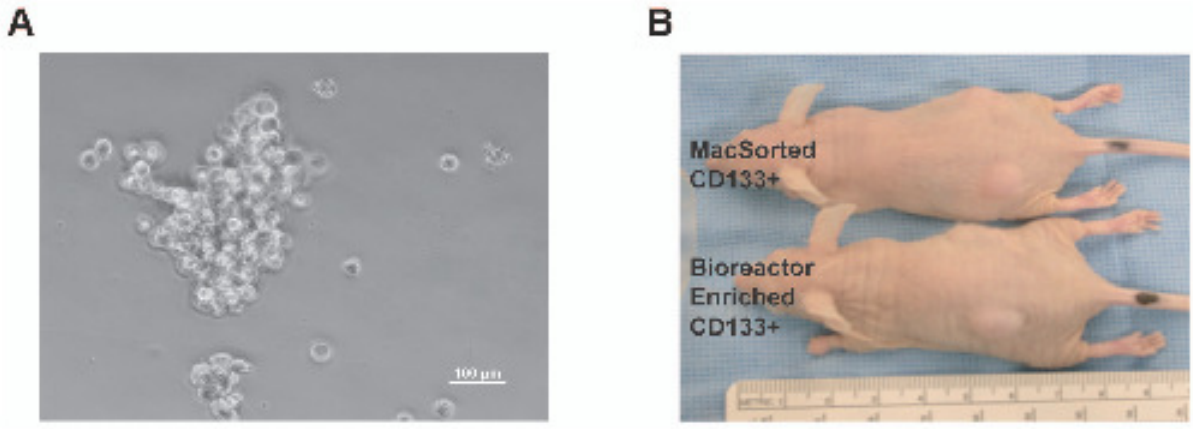


Figure 8. CD133 (+) Ependymoma Cells from Case 1 Grown in a Hydrofocusing Bioreactor form Xenografts in nude Mice.

A) Contrast phase image of a cluster of enriched CSLCs following seven-days of culture in a hydrofocusing bioreactor. B) Immunodeficient nude mice (nu/nu) injected with 1x10² Ependymoma cells MACSorted CD133(+) cells or CD133(+) Ependymoma cells grown in the hydrofocusing bioreactor, with the aid of 100 mL of matrigel in the flank formed a tumor within three months compared to CD133(2) cells. doi:10.1371/journal.pone.0105710.g003

Table 1. Enrichment of CD133+ CSLCs using a hydrofocusing bioreactor.

	CD133+ cells	CD133- cells
Day 0	255,000	245,000
Day 7	3,748,500	159,036
Fold	14.7	−1.54

doi:10.1371/journal.pone.0105710.t001

To verify the tumor-initiating capacity of the HFB grown cells, five immune deficient nude mice/group were injected with a range of $1x10^2$, $1x10^3$, $1x10^4$, and $1x10^5$ cells grown in the HFB (96% CD133+) and compared their growth to an equal number of CD133(+) MACSorted cells and CD133(2) cells for three months. It was observed that both $1x10^2$ MACSorted CD133 (+) cells or the CD133 (+) from the bioreactor grew in all the immune deficient mice injected

and formed a palpable tumor within 12 weeks (Figure 8B). To perform the chemotherapy sensitivity assay a comparable number of cells ($1x10^5$) from the bulk of tumor cells grown as a 2D monolayer or CSLCs enriched in the bioreactor (45) were separately plated into 96 wells plates (n-5 replicas) and were treated for an hour with a series of anticancer drugs at a range of concentrations including the clinically relevant dosage (Table 2). Chemotherapy sensitivity assay was performed using a panel of drugs comprised of Cisplatin, Oxaliplatin, Arabinoside-C, VP-16, Busulfan, Methotrexate, Irinotecan, and Bevacizumab as chosen by the treating oncologist. Sensitivity to chemotherapy was assessed at 48-hours by WST8 viability assay. It was categorized as follows based on the percentage of non-viable cells: responsive (0–40% cell survival), intermediate (40–70% cell survival), and non-responsive (70–100% cell survival). The WST8 assay was conducted three separate times with n=5 well replicas/drug/dose each time.

Table 2. Clinical dose and calculated *in vitro* doses of the various chemotherapies.

	Bevacizumab	**Cisplatin**	**Oxaliplatin**	**Arabinoside-C**	**Irinotecan**	**Busulfan**	**Methotrexate**	**VP-16**
1/10	0.4μM	0.05mM	0.04mM	1.64mM	0.0497mM	0.12mM	2.2μM	0.0339mM
1/100	0.04μM	0.005mM	0.004mM	0.164mM	0.0049mM	0.012mM	0.22μM	0.0033mM
1/1000	0.004μM	0.0005mM	0.0004mM	0.0164mM	0.00049mM	0.0012mM	0.022μM	0.00033mM
Clinical dose	10mg/Kg	75mg/m²	80mg/m²	2g/m²	125mg/m²	150mg/m²	5 mg/m²	100mg/m²
Calculated in vitro dose equivalent to clinical dose	**4μM**	**0.5mM**	**0.4mM**	**16.44mM**	**0.497mM**	**1.22mM**	**22μM**	**0.3397mM**

Results of the chemotherapy sensitivity assay (Figure 9) showed that the Ependymoma cells grown in monolayer and representing the bulk of tumor cells were sensitive to clinically relevant doses of Cisplatin, Irinotecan, Busulfan, and a combination of Irinotecan and Bevacizumab in a statistically significant manner ($p< 0.05$). Interestingly, the CSLCs were

sensitive to a combination of Irinotecan and Bevacizumab ($p< 0.05$), intermediately sensitive to Cisplatin, and Irinotecan, but not sensitive to Busulfan. On the other hand, both the CSLCs and the bulk of tumor cells were not responsive to Methotrexate, Oxaliplatin, Arabinoside-C, and VP-16 (Figure 9). Because of the lack of response to an Oxaliplatin and Etoposide management given in August 2009 (Figure 6B) (which was started prior to receiving the results from the chemotherapy sensitivity assay), in October 2009 the patient underwent a treatment with Bevacizumab and Irinotecan, which was administered every two weeks for six months. In a follow-up MRI scan in May 2010 the patient showed initial disease regression remaining free from disease progression for 18 months (Figure 6C and D). This corresponded to the longest disease progression free period observed in this patient without major de-bulking surgery. Recurrence of tumor growth after 18 months of disease free progression led us to explore novel therapeutic approaches for the treatment of this patient's cancer. In this regard, combination chemotherapy was investigated in order to identify natural compounds that may increase the clinical efficacy of anticancer drugs.

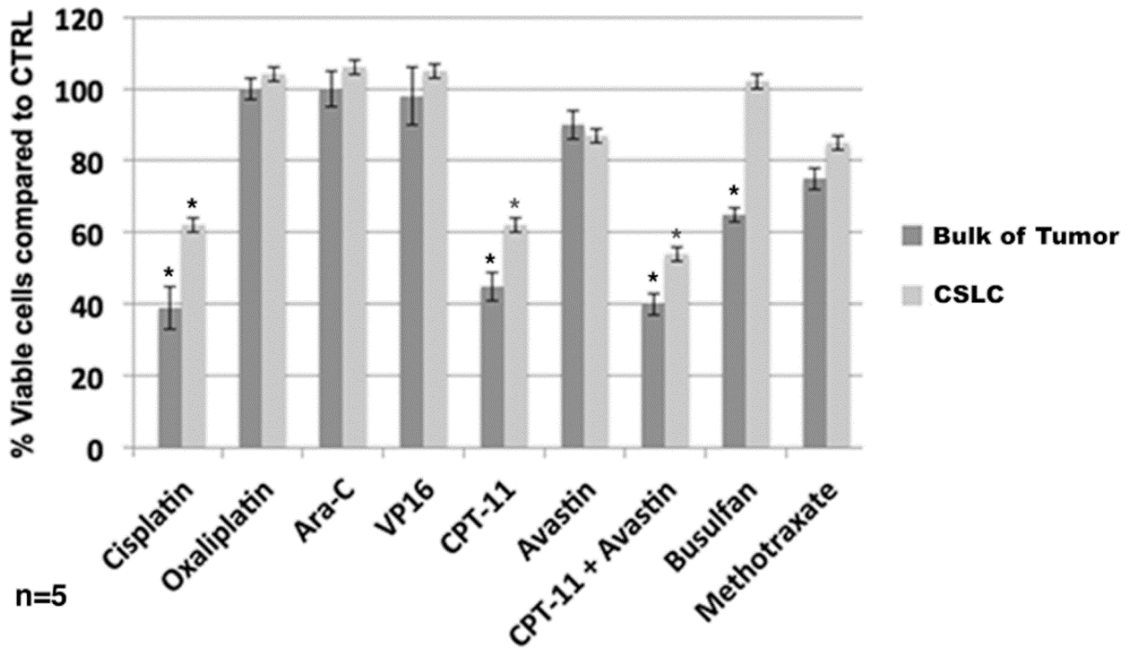


Figure 9. Diagram of Chemotherapy Sensitivity Assay to Assess the Sensitivity to Chemotherapy of Cancer Cells or CSLCs of Ependymoma Case 1 Using a WST-8 Assay.

Bulk of tumor cells or CSLCs were plated with $1x10^3$ cells in each well of a 96-well plate. The treatments were replicated five times in 96-well plates and were challenged for a one-hour pulse with a panel of anticancer drugs indicated by the oncologist. A WST-8 assay was performed 48-hours following chemotherapy treatments to assess cell viability. The data above is plotted in bar graph and results were determined as responsive (0–40% cell viability), moderately responsive (40–70% cell viability), and non-responsive (70–100% cell viability). Light grey bars represent sensitivity of CSLCs to chemotherapy with respect to negative untreated control cells. Dark grey bars represent sensitivity of bulk of tumor cells to chemotherapy with respect to negative untreated control cells. Anticancer drugs tested indicated at the bottom of the diagram. Statistical analysis of the results was performed using one-way ANOVA. Asterisks indicate p values of less than 0.05. doi:10.1371/journal.pone.0105710.g004

Benzyl isothiocyanate (BITC) has been shown (71,72) to increase the chemosensitivity of cancer cells. This study recently observed (Figure 10) that BITC increases specifically the chemosensitivity of CD133 positive cancer cells. Because the primary Ependymoma cells of Patient 1 displayed a high percentage of cells positive to CD133, the hypothesis that BITC could increase their chemosensitivity to Irinotecan and Bevacizumab was tested. Increasing concentrations of BITC ranging from 2.5 mM to 20 mM decreased the viability of CD133(+)

Ependymoma cells of Patient 1 from 90% to 62% in a statistically significant manner (Figure 11A). Chemotherapy sensitivity assay also determined that the combination of Irinotecan and a non-toxic concentration of 10 mM BITC reduced the viability of the Ependymoma cells from 60% to 40% (over 40% more chemosensitive compared to non BITC treated cells) (Figure 11B). Additionally, the combination of Irinotecan and Bevacizumab with BITC reduced even further the viability of the Ependymoma cells to 30% (Figure 6B). The patient was treated with Irinotecan and Bevacizumab, but this time with the combination of two capsules/day of a Triple Action Cruciferous Vegetable Extract containing high concentration of BITC (LifeExtension, http:// www.lef.org), for two months. Following the combination therapy of Irinotecan, Bevacizumab and the supplement of cruciferous vegetable extract there was a 4 cm regression (which corresponds to a 50% regression) of the lesions in the thoracic and the cervical area [compare Figure 6C (at recurrence) to Figure 11D (following therapy)]. Additionally, it was reported that the patient was able to tolerate the entire course of Irinotecan and Bevacizumab chemotherapy regimen with less fatigue and better tolerance to cold.

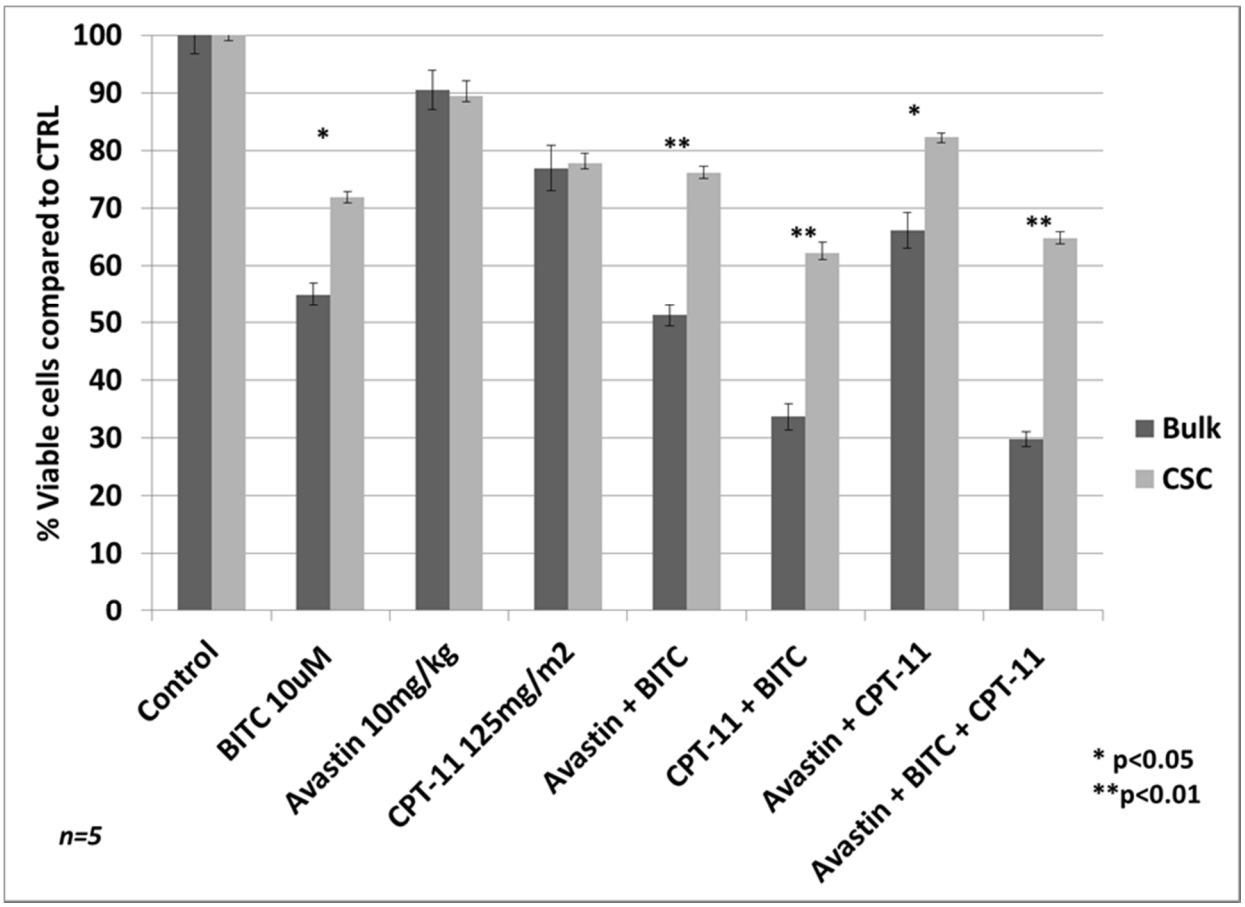
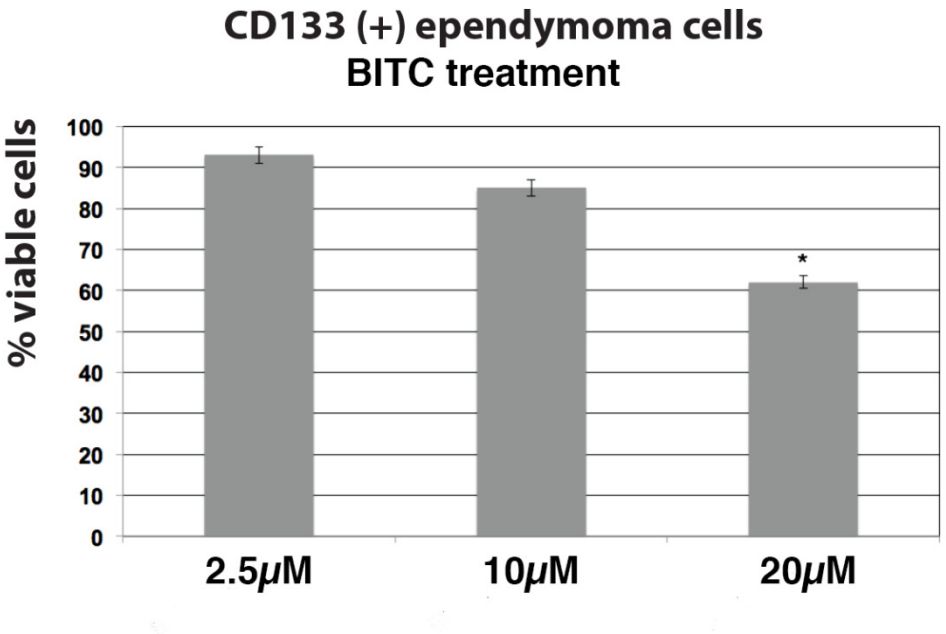


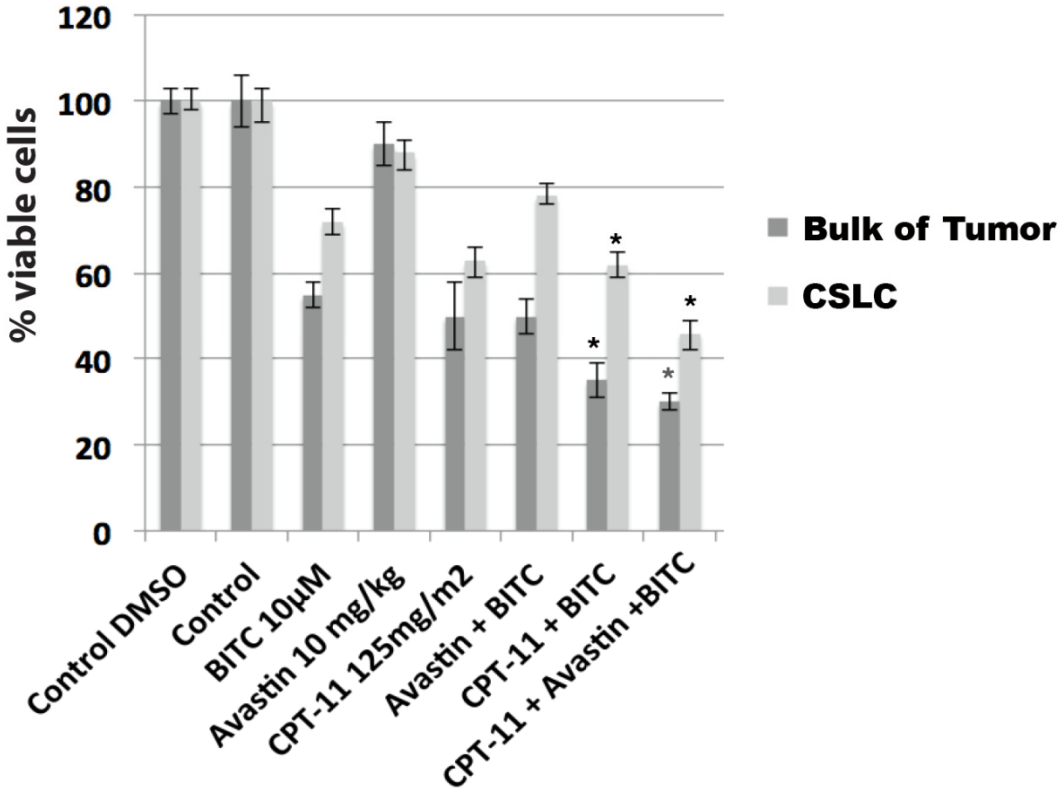
Figure 10. Diagram of Chemotherapy Sensitivity Assay to Assess the Sensitivity to BITC and Chemotherapies of Cancer Cells or CSLCs of Ependymoma Case 1.

1x10³ bulk of tumor cells or CSLCs plated in five replicas into 96-well plates were treated with 10uM of BITC for 24 hours before being challenged for a one-hour pulse with CPT-11, Avastin or a combination of the two. A WST-8 assay was performed 48 hours following BITC treatments to assess cell viability. Data is plotted in bar graph as responsive (0–40% cell viability), moderately responsive (40–70% cell viability), and non-responsive (70–100% cell viability). Light grey bars represent sensitivity of CSLCs to chemotherapy with respect to negative untreated control cells. Dark grey bars represent sensitivity of bulk of tumor cells to chemotherapy with respect to negative untreated control cells. Anticancer drugs tested indicated at the bottom of the diagram. Statistical analysis of the significance of the results was performed with a one-way ANOVA. Single asterisks indicate p values of less than 0.05. Double asterisks indicate p values of less than 0.01.

A


CD133 (+) ependymoma cells
BITC treatment
% viable cells
100
90
80
70
60
50
40
30
20
10
0
*
2.5μM
10μM
20μM

B


120
100
80
60
40
20
0
% viable cells
*
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Bulk of Tumor
CSLC
Control DMSO
Control
BITC 10μM
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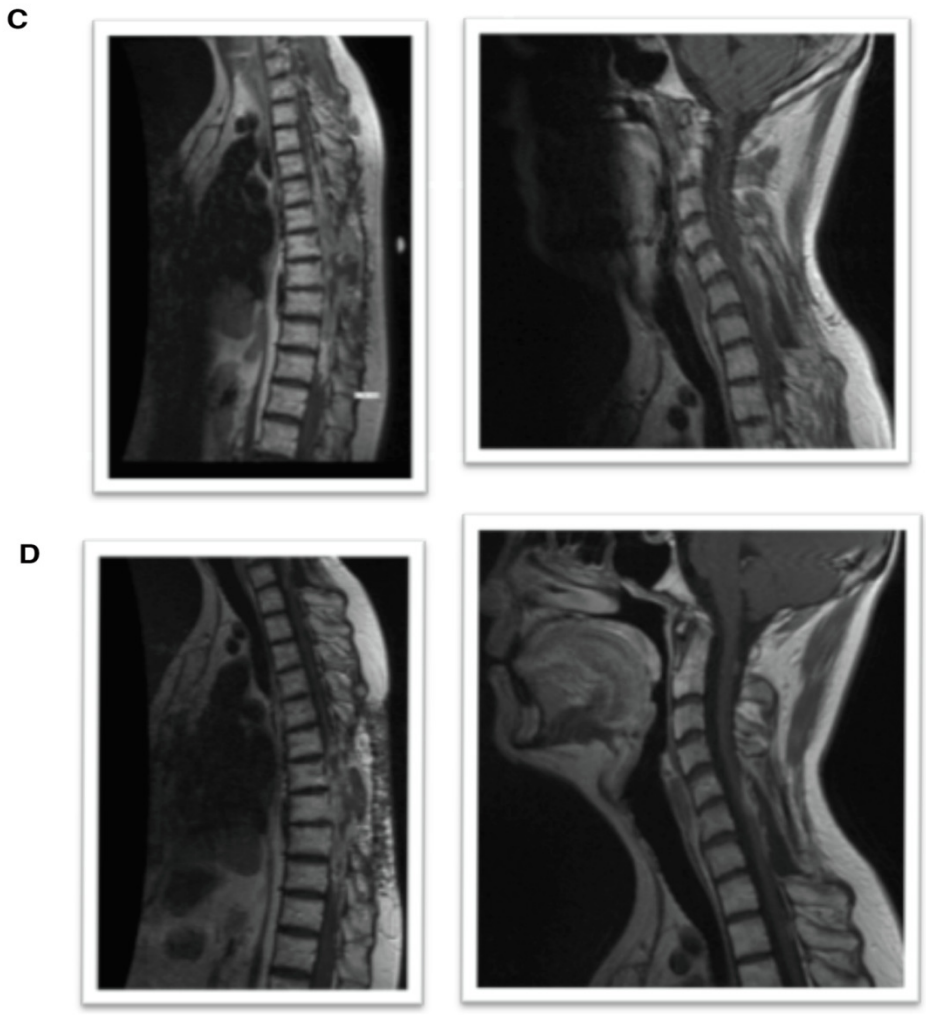

Figure 11. Diagram of Chemotherapy Sensitivity Assay and MRI Images of Cervical and Thoracic Spine following Integrated Therapy of Ependymoma Case 1.

A) 1x10³ CSLCs plated in five replicas into 96-well plates were challenged for a one-hour pulse with 2.5, 10, and 20 mM BITC. A WST-8 assay was performed 48 hours after treatments to assess cell viability. B) 1x10³ CSLCs plated in five replicas into 96-well plates were challenged for a one-hour pulse with 10 mM BITC followed by a one-hour pulse with 0.5 mM CPT-11. A WST-8 assay was performed 48 hours following chemotherapy treatment to assess cell viability. Data is plotted in bar graph as responsive (0–40% cell viability), moderately responsive (40–70% cell viability), and non-responsive (70–100% cell viability). Light grey bars represent sensitivity of CSLCs to chemotherapy with respect to negative untreated control cells. Dark grey bars represent sensitivity of bulk of tumor cells to chemotherapy with respect to negative untreated control cells. Statistical analysis of the significance of the results was performed with a one-way ANOVA. Asterisks indicate p values of less than 0.05. C) 2012 MRI of the cervical and thoracic spine showing recurrence after an 18 months progression free period. D) 2012 MRI of the cervical spine showing marked tumor regression of the thoracic spine lesion following combined treatment with Irinotecan (CPT11), Bevacizumab (Avastin), and BITC supplementation. doi:10.1371/journal.pone.0105710.g005

The efficacy of chemotherapies screened *in vitro* by the chemotherapy sensitivity assay were tested on the Ependymoma cells of Patient 1 that were xenografted in a NOD-Scid mouse model (Figure 12 A and B). Ten athymic NOD-Scid mice were injected in the flank with $1x10^6$ Ependymoma cells mixed to 100 uL of matrigel (BD Biosciences, San Jose, CA) and tumors were grown for ten weeks or until 100 mm^3. Randomized mice were treated with weekly intraperitoneal (i.p.) injections of the different treatment arms for four weeks and were observed for four more weeks. Group #1 serving as a control received i.p. sterile saline injections. Groups #2–5 were the experimental groups, which received i.p. injections of the least effective chemotherapy, or the most effective, the second most effective, and the most effective combinatorial chemotherapy, as determined by the *in vitro* chemotherapy sensitivity assay. Interestingly, the tumor xenografts in the Scid mice injected with the least effective chemotherapy as determined by the *in vitro* chemotherapy sensitivity assay grew faster than saline control injected mice (Figure 12A). As expected, tumor regression was observed in Scid mice treated with the most effective, the second most effective, and the most effective combinatorial chemotherapy as determined by the *in vitro* chemotherapy sensitivity assay, confirming the clinical observation that Irinotecan and Bevacizumab are more effective anticancer drugs in this individual patient. Mice weight was measured weekly (Figure 12B).

The hypothesis was tested further by mice that were failing a chemoresistant treatment could be rescued by switching them to a more sensitive treatment as determined by the *in vitro* chemotherapy sensitivity assay. Mice that were failing an Oxaliplatin therapy regimen were taken off Oxaliplatin at week 16 and were treated for four weeks with a combination of Irinotecan and Bevacizumab. As expected, mice treated with Irinotecan and Bevacizumab

showed a regression of the xenografted tumor compared to the control mice injected with saline solution (Figure 12C) confirming once again the previously observed clinical data.

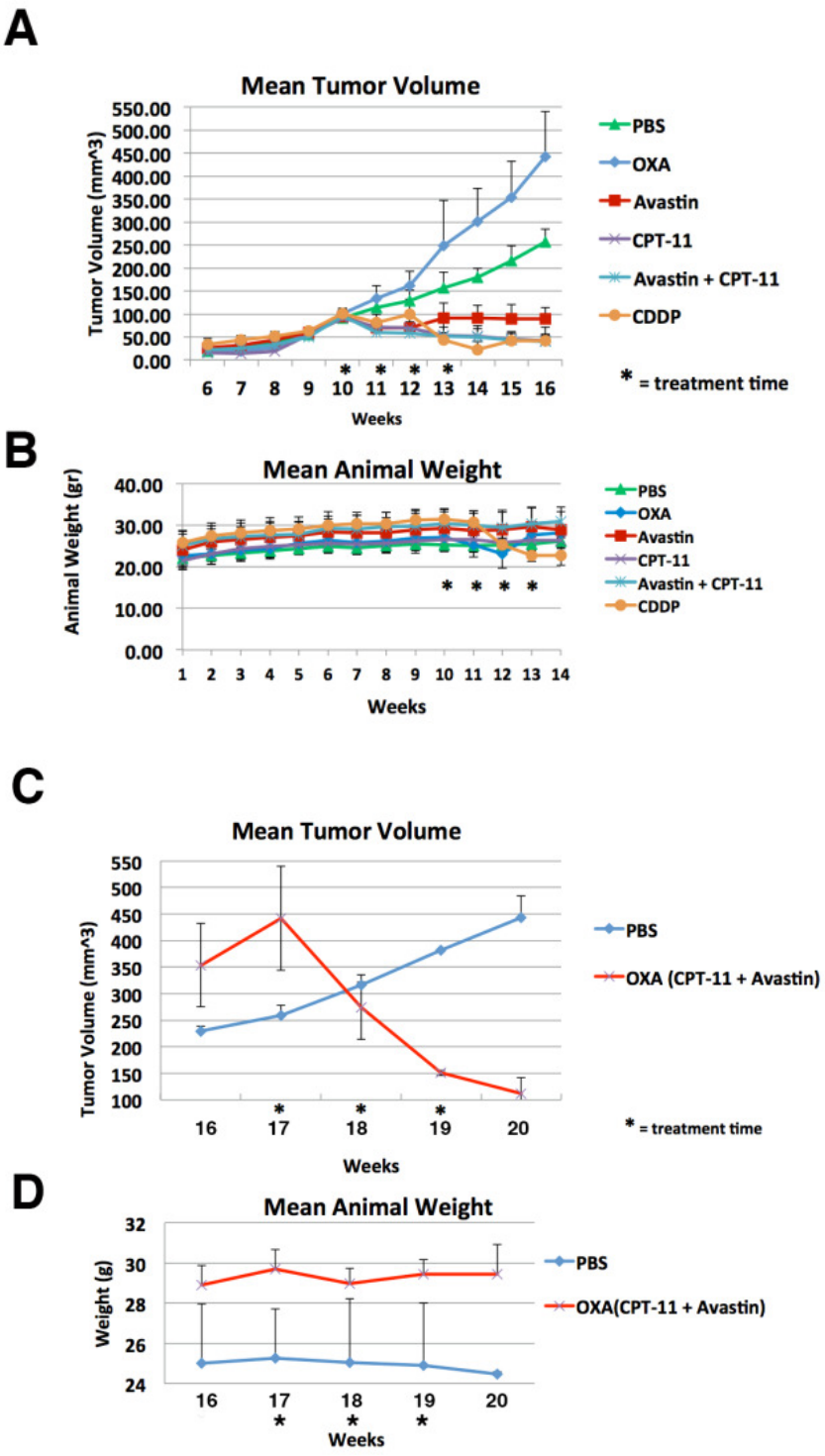

Figure 12. Mean Tumor Volume and Mean Tumor Weight of Patient Derived Xenografts (Ependymoma Case 1) Treated with i.p. Injection of Anticancer Drugs.

A) Line diagram of the mean volumes in mm³ (±SD) from week 6–16 of ten patient derived xenografted tumors in NOD-Scid mice following four weeks of treatment with various anticancer drugs. The mean tumor volumes are indicated on the ordinate. Asterisks indicate weeks in which treatment was performed.

On the right are indicated the different treatment arms. PBS: saline solution, negative control. OXA (Oxaliplatin); Avastin (Bevacizumab); CPT-11 (Irinotecan); CDDP (Cisplatin). B) Line diagram of the mean weight in grams (±SD) of ten NOD-Scid mice-bearing patient derived xenografted tumors following four weeks of treatment with various anticancer drugs. The mean tumor weights are indicated on the ordinate. Asterisks indicate weeks in which treatment was performed. On the right are indicated the different treatment arms. PBS: saline solution is negative control. OXA (Oxaliplatin); Avastin (Bevacizumab); CPT-11 (Irinotecan); CDDP (Cisplatin). C) Line diagram of the mean volumes in mm^3 (±SD) from week 16 to 20 of the ten patient derived xenografted tumors in NOD-Scid mice that failed Oxaliplatin therapy (weeks 6–16 in panel A), following three weeks of treatment with Irinotecan and Bevacizumab. The mean tumor volumes are indicated on the ordinate. Asterisks indicate weeks in which treatment was performed. On the right are indicated the different treatment arms. PBS: saline solution, negative control. OXA (CPT11+Avastin): mice that failed Oxaliplatin and were then treated with Irinotecan and Bevacizumab. D) Line diagram of the mean weight in grams (±SD) of the ten NOD-Scid mice-bearing patient derived xenografted tumors following three weeks of treatment with Irinotecan and Bevacizumab. The mean tumor weights are indicated on the ordinate. Asterisks indicate weeks in which treatment was performed. On the right are indicated the different treatment arms. PBS: saline solution, negative control. OXA (CPT11+Avastin): mice that failed Oxaliplatin and were then treated with Irinotecan and Bevacizumab. doi:10.1371/journal.pone.0105710.g006

Ependymoma Patient 2

Patient 2 was a five-month-old female with an aggressive brain tumor that was surgically removed in April 2012. The tumor was diagnosed as an Anaplastic Ependymoma, WHO grade III with low-grade mitosis-poor areas and high cellular tissue with mitosis and high MIB-1 rate.

A biopsy from the surgically removed tumor was placed in RPMI-1640 sterile media and the tissue was dissociated in the laboratory into a single-cell suspension with the use of a GentleMACS tissue dissociator (Miltenyi, Aubourn, CA) as done in the previous case. The single-cell Ependymoma suspension was plated in RPMI-1640 in the presence of 5% irradiated, heat inactivated, defined fetal bovine serum, streptomycin and penicillin and cells were cultured as a monolayer for 15 days. Cells were immunophenotyped by flow cytometry using antibodies against CD34, CD38, CD44, CD133, Nanog, and CXCR4. The Ependymoma cells were found positive to Nanog (13%), CD133 (47.5%), CD44 (65.5%), and CXCR4 (89.7%) when compared to an isotype control antibody. A double staining of CD34 and CD38 showed the presence of 4.6% of the cells CD34+/CD38+, and 47.3% CD34+/CD38- cells.

The chemotherapy sensitivity assay performed on the bulk of the Ependymoma cells and on the CSLCs showed resistance to all of the tested chemotherapy drugs (Figure 13). Patient 2 received complex chemotherapy with six cycles of Vincristine, Carboplatin, Cyclophosphamide, Etoposide, and Cisplatin in various combinations, however the tumor rapidly progressed and proton beam therapy was recommended. The tumor did not respond to the various anticancer drugs and radiation therapy and the patient expired after nine months.

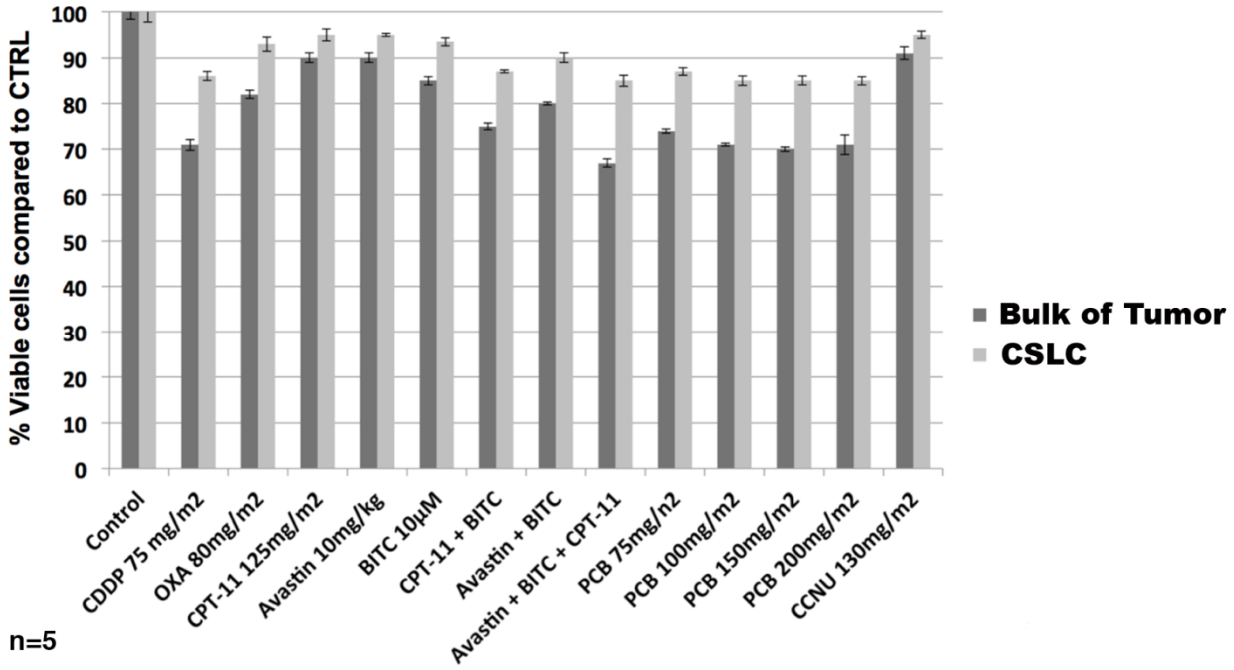

Figure 13. Diagram of Chemotherapy Sensitivity Assay to Assess the Sensitivity to Chemotherapy of Cancer Cells or CSLCs Using a WST-8 Assay on Ependymoma Case 2.

1×10^3 bulk of tumor cells or CSLCs plated in five replicas into 96-well plates were challenged for a one-hour pulse with a panel of anticancer drugs indicated by the oncologist. A WST-8 assay was performed 48 hours following chemotherapy treatments to assess cell viability. Data is plotted in bar graph as responsive (0–40% cell viability), moderately responsive (40–70% cell viability), and non-responsive (70–100% cell viability). Light grey bars represent sensitivity of CSLCs to chemotherapy with respect to negative untreated control cells. Dark grey bars represent sensitivity of bulk of tumor cells to chemotherapy with respect to negative untreated control cells. Anticancer drugs tested indicated at the bottom of the diagram. Statistical analysis of the significance of the results was performed with a one-way ANOVA. Asterisks indicate p values of less than 0.05. doi:10.1371/journal.pone.0105710.g007

DISCUSSION

Treatment for Ependymoma is often a combinatorial approach that includes surgery, radiation therapy, and chemotherapy. Although chemotherapy has been used extensively in the treatment management of Ependymomas, this therapeutic modality is often reserved for patients with residual tumor after surgery and for children younger than three years of age in an attempt to delay radiation therapy. Recently, the role of chemotherapy in the treatment of Ependymoma has diminished because (1) chemotherapy fails to delay the need for radiation therapy for a meaningful period of time; (2) tumors that progress during chemotherapy do not respond as well to subsequent irradiation; and (3) the combination of chemotherapy and irradiation does not improve overall survival (59,73).

It is not entirely clear why there is not an improved survival with chemotherapy (63), therefore investigation and development of novel strategies and integrated therapies are required to find more effective treatments for this type of tumor.

One of our patients was diagnosed with recurring undifferentiated intradural-extramedullary spinal Ependymoma, WHO grade III, with a distinctive sensitivity to chemotherapy who has been followed up for five years following chemotherapy sensitivity assay. The second patient was also diagnosed with recurring Ependymoma, WHO III but was found not sensitive to any of the chemotherapies tested and rapidly progressed.

Resistance to chemotherapy severely compromises its effectiveness. The development of resistance is a major problem for patients, researchers, and clinicians who rely on conventional cytotoxic agents for the treatment of cancer.

Despite the fact that several treatments for Ependymoma are currently available, this remains a poorly treated disease (74-78). Surgery plus postoperative radiotherapy represents the

standard treatment for patients with grade III (Anaplastic) Ependymomas (78,79). Additionally, surgery has been demonstrated to be associated with significant improvements in overall survival time for patients with all stages of ependymal tumors (80-84); however, a total resection is not always achieved. Overall prognosis is improved when the entire tumor can be removed and there are no other neural axis metastases (85). Therefore, in cases in which the Ependymoma is multifocal, metastatic, incompletely resected, or particularly aggressive, it is imperative to find the most effective alternative treatment to surgery available.

Administration of ineffective anticancer therapy is associated with unnecessary toxicity and development of resistant clones. Each time patients are treated, they have a chance of relapse and their cancer may become more resistant to therapy. Presently used anticancer drugs have a high rate of failure and cell culture chemotherapy testing is being used to identify which drugs are more likely to be effective against a particular tumor type. Measuring the response of the tumor cells to drug exposure is valuable in any situation in which there is a choice between two or more treatments. In virtually all situations in cancer chemotherapy, the goal is cure or palliation. This kind of testing can assist in individualizing cancer therapy by providing information about the likely response of an individual patient's tumor to proposed therapy. Many attempts have been made over the years to develop an ex-vivo anti-cancer test that can provide clinically relevant treatment information, but all the efforts have been directed toward the bulk of tumor cells (86-92).

In the recent past, chemotherapy testing has been performed on cancer cells from patients without prior separation and enrichment of the CSLCs from the bulk of tumor cells (9-12,87,93-98).

Knowing which chemotherapy agents the patient's bulk of tumor cells, as well as the CSLCs, are resistant to is very important. Then, these options can be eliminated, thereby avoiding the toxicity of ineffective agents. Choosing the most effective agent can help patients to avoid the physical, emotional, and financial costs of failed therapy and experience an increased quality of life.

The chemotherapy sensitivity assay used in this study measures for the first time the survival of CSLCs and bulk of tumor cells cultured from human cancer biopsies to chemotherapeutic agents. The advantage of the chemotherapy sensitivity assay is to aid the oncologists in selecting the most appropriate chemotherapy regimen on an individual basis especially when a number of equivalent options are available. The chemotherapy sensitivity assay allows various available chemotherapy drugs, which are part of standard of care to be tested for efficacy against the cancer stem cells, as well as the bulk of tumors.

For Patient 1, affected by a recurring anaplastic Ependymoma, the chemotherapy sensitivity assay determined on both bulk of tumor cells and CSLCs, that the most effective treatments were either Irinotecan and Bevacizumab or Cisplatin. Interestingly, although the entire regimen containing Irinotecan and Bevacizumab could not be completed, the patient showed an initial regression of the disease and remained free from disease progression for 18 months, which corresponded to the longest disease progression free period in this patient.

Following up on the recurrence after the 18 month of progression free interval observed, repeated testing was performed using the chemotherapy sensitivity assay on the combination of several drugs and nutritional supplements, among which Benzyl isothiocyanate (BITC). Numerous studies have indicated that Isothiocyanates (ITCs) induce robust anti-cancer effects

(72,99,100). ITCs are derived naturally from glucosinolates, which are found at high concentrations in vegetables from the Cruciferae family (71,72).

Cruciferous vegetables, which produce ITCs, include broccoli, Indian cress, cabbage, Brussel sprouts, and watercress (101). ITCs are of interest as anticancer molecules because of their ability to target many of the aberrant pathways associated with cancer development. However, among the numerous ITCs identified, only a few of them appear to have anti-carcinogenic properties (102).

Interestingly, BITC has been previously shown to increase the chemosensitivity of bulk of tumor cells (71,72), but not of CSLCs. In this study it has been observed that BITC can increase specifically the chemosensitivity of cells that are highly positive for CD133 (data not shown), a marker used to identify CSLCs in tumors of the nervous system. Since the primary Ependymoma cells of Patient 1 displayed a high percentage of cells positive to CD133, it was advantageous to do further testing of the hypothesis that BITC could increase the patient's chemosensitivity.

Interestingly, as demonstrated here, for the first time, that the combination of Irinotecan and BITC increased the chemosensitivity of the bulk of tumor cells and of the CSLCs cultured from the Ependymoma of Patient 1. There was a clinically significant regression of the lesion in the cervical area as well as regression of other lesions at the thoracic level following a combined treatment with Irinotecan, Bevacizumab, and BITC.

Noteworthy and as expected, regression was observed of the NOD-Scid mice xenografts treated with the most effective, the second most effective, and the most effective combinatorial chemotherapy as determined by the *in vitro* chemotherapy sensitivity assay. In a model of patient derived xenografts this confirms the clinical observation that Irinotecan and Bevacizumab are

more effective anticancer drugs for this individual patient. Interestingly, the tumor xenografts in the Scid mice injected with the least effective chemotherapy as determined by the *in vitro* chemotherapy sensitivity assay grew faster than saline control injected mice. It is not known why the tumor xenografts in mice injected with Oxaliplatin grew faster than saline control injected mice, but speculated that because the patient was treated with Oxaliplatin prior to the chemotherapy sensitivity assay biopsy, it had selected cellular clones that are resistant to it and that manifest a growth advantage in its presence.

Furthermore, mice that did not show regression to Oxaliplatin treatment, which mimics the clinical scenario of this particular patient, were rescued by switching them to a more effective treatment (Irinotecan and Bevacizumab) as determined by the *in vitro* chemotherapy sensitivity assay. As expected, in this rescue animal model the mice treated with a combination of Irinotecan and Bevacizumab showed a regression of the patient derived xenografted tumors compared to control mice injected with saline solution confirming once again the previously observed clinical data.

Unfortunately, the second case of Ependymoma presented could not benefit from any combined therapy that was proposed indicating that although affected by the same type of tumor response to chemotherapy was different.

This is the first report on the clinical relevance of this novel chemosensitivity assay that measures the sensitivity of bulk of tumor cells and CSLCs to chemotherapy, which has the objective to decrease unnecessary toxicity while increasing the benefit of cytotoxic therapy for patients affected by malignant tumors.

Although the chemotherapy sensitivity assay results on these two cases of Ependymoma showed clinical relevance, a larger study with different histological tumor types is needed to

determine the prognostic accuracy of this assay. This laboratory is currently conducting a brain and spine malignant tumor phase-I clinical trial in which 33 patients have accrued in the past three years to study the feasibility of this new assay in predicting the most effective chemotherapy regimen to improve patients' outcomes by assessing the vulnerability to chemotherapy of the CSLCs.

CHAPTER 3 CASE STUDY OF GLIOBLASTOMA

ABSTRACT

Among the different types of central nervous system (CNS) tumors, glioblastoma multiforme (GBM) is the most aggressive and malignant primary brain tumor, exhibiting the highest mortality rate among the glioma subtypes, with a median survival of 14 months. GBM is persistently chemoresistant with substantial rates of reoccurrence. Most patients with GBM are treated with surgery followed by chemo-radiation therapy either at the time of initial diagnosis or at tumor recurrence.

This study was designed to investigate an *in vitro* chemotherapy sensitivity assay, which measures the sensitivity of CSLCs as well as the bulk of tumor cells to a variety of chemotherapy agents for two Glioblastoma (GBM) patients. Patient 1 was a 56-year old male affected by a GBM IDH-1 mutant. This patient was found 50% sensitive to the treatment of Temodar (TMZ) on the bulk of the tumor, but resistant to TMZ when treating the CSC population. Six months after initial therapy (surgery, TMZ and radiation) a recurrence was observed. Patient 2 was a 35-year old male with GBM IDH-1 wild-type. This patient was also treated with surgery followed by TMZ and radiation and at the six months follow up was found in remission. This event was predicted by the chemotherapy sensitivity assay that indicated a response of both CSLC and Bulk of tumor cells to TMZ.

The results of the *in vitro* assays were confirmed by the use of mice bearing patient derived xenografts treated with the drugs screened by the chemotherapy sensitivity assay. The animal data was found to be in accordance with the data from both the patients' outcome and the *in vitro* studies. This assay demonstrated again that patients with the same histological stage and

grade of cancer may not always respond to the same standard-of-care clinical treatment, suggesting that a chemotherapy sensitivity assay which measures the sensitivity of CSLCs as well as the bulk of tumor cells to a variety of chemotherapy agents could lead to more effective and personalized anticancer treatments in the future.

INTRODUCTION

Glioblastoma (GBM) or WHO grade IV malignant glioma is diagnosed in about 10,000 patients a year, making it the most common primary brain cancer in adults (46,52,103-105). Standard treatment usually consists of surgery followed by chemo-radiotherapy allowing for an average survival rate of only 14 months (3,52,103-108). The first line chemotherapeutic agent used is an oral medication called Temozolomide (Temodar, TMZ); it is an alkylating agent, disrupting DNA replication (3,6,52,104-107).

Though advancements have been made in the treatment of GBM most patients have recurrence within a year (107). This poor prognosis has been associated with GMB heterogeneity, and the CSLC that initiate and maintain this disease (3,46,52,104). Several studies have identified biomarkers of anticancer drug resistance as well as tumor initiation (3,6,46,52,104). The subpopulation of CD133 positive cells has been shown to contain tumor initiator cells for GBM (3,46). This evidence supports the Cancer Stem Cell Hypothesis, and indicates that targeting this population would lead to novel treatments that significantly improve patient survival (46,52).

There is an indication that some factors of the patient that may influence the survival rate and treatment resistance in patients include the status of the IDH1 gene and the methylation status of the O6-methylguanine-DNA-methyltransferase gene (MGMT) (3,6,108). The IDH-1 gene encodes for cytosolic NADP+-dependent isocitrate dehydrogenase, which is involved in the control of oxidative cellular damage. It has been found that patients that have a mutation in the IDH-1 gene have better prognosis (108). The IDH-1 gene encodes for an enzyme located in the cytoplasm and in peroxisomes that catalyzes the oxidative carboxylation of isocitrate to α-ketoglutarate, which allows the reduction of NADPH. The production of NADPH is essential for

the regeneration of reduced glutathione, which functions as the main antioxidant in mammalian cells and promotes resistance to apoptosis. IDH-1 therefore plays a prominent protective role against oxidative damage induced by ROS via the regeneration of reduced glutathione (109).

In this context, mutation of IDH-1 appears paradoxical: on one hand, mutant IDH-1 cells may be more sensitive to genetic instability caused by an oxidative environment, and thus IDH-1 mutations may contribute to tumor development, but on the other hand, mutant IDH-1 cells are less protected against oxidative cellular damage (109).

This study investigated the chemosensitivity of Bulk of tumor cells and CSLC in two primary cell lines generated from two patients who were newly diagnosed with GBM and never treated before. GBM Patient 1 was found IDH-1 mutant and in GBM Patient 2, IDH-1 was found to be wild-type (Wt).

MATERIALS AND METHODS

Cell Culture and Reagents

All cell culture was performed using previously established methods (50). All clinical grade chemotherapeutic agents were acquired as a gift from Edwards Comprehensive Cancer Center.

Patients

Case 1 is a 56-year-old male patient diagnosed with an IDH-1 mutant GBM. Case 2 is a 35-year-old male with a IDH-1 wild type GBM. Chemotherapy sensitivity assay was performed after obtaining patient's written informed consent in accordance with the ethical standards of the Helsinki Declaration (1964, amended most recently in 2008) of the World Medical Association. Any information, including illustrations, has been anonymized. The Marshall University Institutional Review Board (IRB) has approved this research under the protocol #326290. Ethics committees/ IRB at Marshall University approved this consent procedure.

Three-Dimensional Bioreactor CSCs Culture

A hydrodynamic focusing bioreactor (HFB) (Celdyne, Houston TX) was used as previously described to selectively proliferate the CD133 (+) cancer stem-like cells (45).

Cell Sorting

Up to 10 million cells were sorted for CD133+ cells by a magnetic-activated cell sorting (MACS) system as described before (50).

Flow Cytometry Studies

Cells were analyzed by the antigenic criteria using anti-CD34 (Milteny Biotech, Auburn, CA), -CD38 (Milteny Biotech, Auburn, CA), -CD44 (BD Bioscience, Sparks, MD), -CD133/2 (prominin1) (Milteny Biotech, Auburn, CA), -CXCR4 (BD Bioscience, Sparks, MD), -Oct3/4 (BD Bioscience, Sparks, MD), and –Nanog (BD Bioscience, Sparks, MD). All methods were performed as previously described (50).

Chemotherapy Sensitivity Assay

Sensitivity to chemotherapy was assessed using the viability assay MTT on 10,000 cells plated in four replicas into 96-well plates. Briefly, equal numbers of bulk tumor cells grown in a monolayer and CSLCs grown in the bioreactor, were counted and seeded separately in 96-well dishes and incubated at 37°C for 24 hours. The cells were then challenged with a one-hour pulse of a panel of individual anticancer drugs as chosen by the oncologist to mimic the average clinical chemotherapy infusion schedule.

An MTT assay was performed 24 hours following chemotherapy treatment to assess cell viability. A dose response chart was developed in which samples were scored as responsive (100–60% cell death), intermediate (60–30% cell death), and non-responsive (0–30% cell death).

Animal Study

All animal studies were conducted following approval from the Marshall University IACUC, protocol #373017. The effects of chemotherapies screened *in vitro* by the chemotherapy sensitivity assay were tested on human tumor biopsies that were xenografted in the flank of a NOD-Scid mouse model. This was performed using a previously established method (50).

Animals were euthanized following the current guidelines established by the latest Report of the AVMA Panel on Euthanasia using CO2 inhalation and asphyxiation followed by cervical dislocation.

Statistical Analysis

Statistical analysis was performed as previously established (50). *P-values* of less than 0.05 were considered statistically significant.

RESULTS

Patient 1

The patient is a 56-year-old male who had a craniotomy on April 18th 2012. The pathology report came back as Glioblastoma IDH-1 mutant. Appropriate informed consent was signed and at the time of the debulking surgery, a sterile biopsy was taken to assess the sensitivity of the tumor cells (bulk of tumor and CSLCs) toward standard-of-care chemotherapy drugs using a chemotherapy sensitivity assay. The biopsy was placed in collection/transport media and tissue was dissociated in the laboratory into a single-cell suspension with the use of a GentleMACS tissue dissociator (Miltenyi, Aubourn, CA). The single-cell GBM suspension was plated as previously reported (50) and cells were cultured as a monolayer for 15 days. Cells were immunophenotyped by flow cytometer using antibodies against CD133, CD34, CD38, CD44, CD24, CXCR4, OCT3/4, and Nanog (Figure 14).

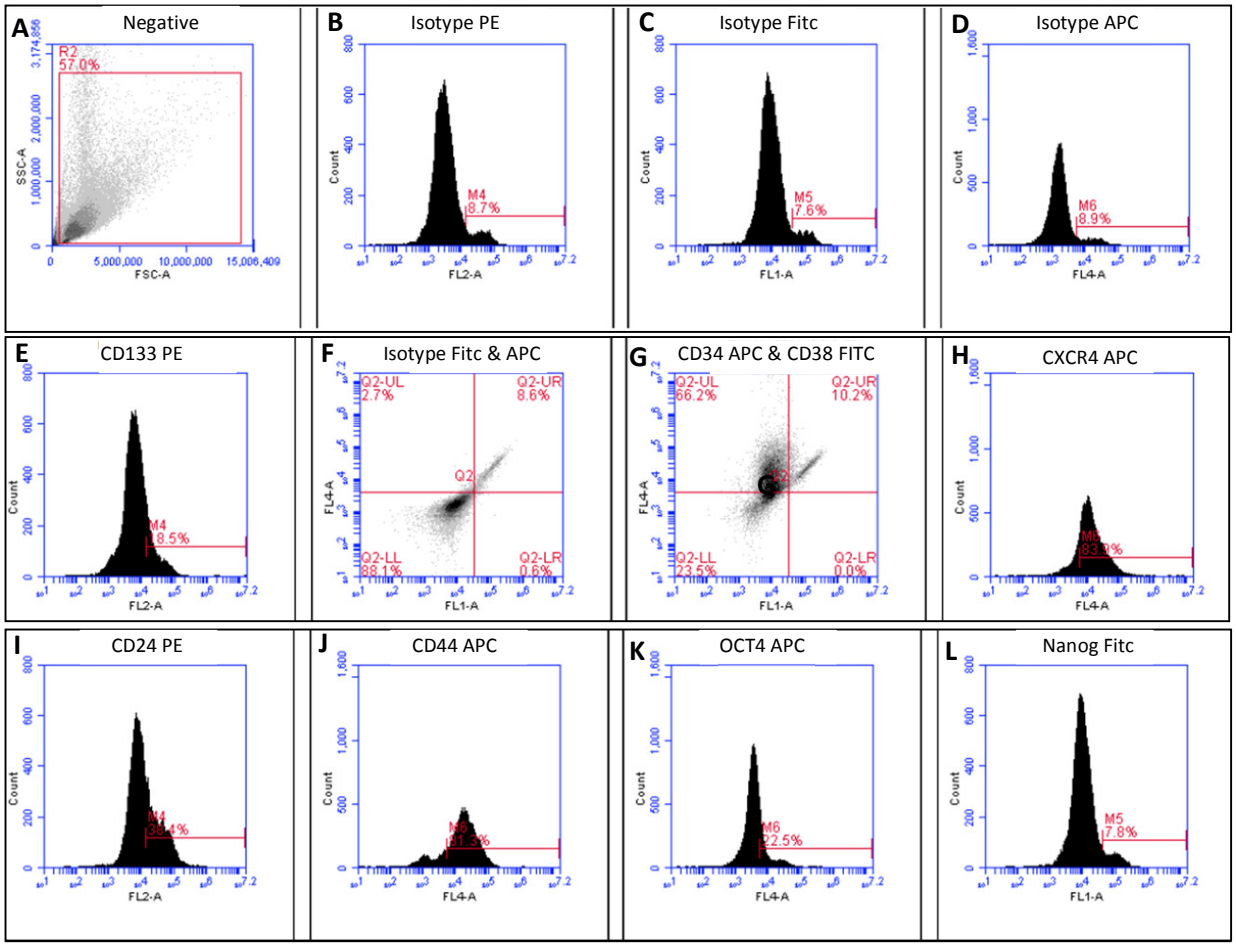

Figure 14. Flow Cytometer Data for GBM Patient 1.

FL1-A is the filter used to measure Fitc, FL2-A is the filter used to measure PE, FL4- A is used to measure APC. A) Negative control showing FSC and SSC on cells not labeled with any antibody. B) Isotype control for PE. C) Isotype control for Fitc. D) Isotype control for APC. E) CD133/2-PE labeled cells. F) Isotype control for Fitc vs APC. G) CD38-Fitc Antibody vs CD34-APC. Upper left quadrate is CD34+/CD38-, upper right quadrant is CD34+/CD38+, lower left quadrant is CD34-/CD38-, and lower right quadrant is CD34-/CD38+. H) CXCR4-APC labeled cells. I) CD24-PE labeled cells. J) CD44-APC labeled cells. K) OCT 3/4-APC labeled cells. L) Nanog-Fitc labeled cells.

	CD133	CD44	CD24	CXCR4	OCT3/4	Nanog	CD34+/CD38-
Percent	9.8	72.4	29.7	75.0	13.6	0.2	63.5

Table 3. Flow Cytometer Data on GBM Patient 1.
Shows the percent of cells tested that are positive for each biomarker compared to the Isotype controls.

The GBM cells were found positive to CD133 (9.8%), CD44 (72.4%), CD 24 (29.7%), CXCR4 (75.0%), OCT3/4 (13.6%), and Nanog (0.2%) when compared to an isotype control antibody (Table 3). A double staining of CD34 and CD38 showed the presence of 1.6% of the cells CD34+/CD38+, and 63.5% CD34+/CD38- cells (Table 3). To expand the CSLC population of CD133+ cells from the GBM primary culture, the cells were cultured as previously described (50). $1x10^6$ GBM cells were cultured in the HFB to isolate the CSLCs for chemotherapy sensitivity assay. When placed directly in non-attaching dish they maintained a cluster assembly (Figure 15A) and would repopulate the morphologically heterogeneous GBM culture after ten days in a regular tissue culture dish (Figure 15B).

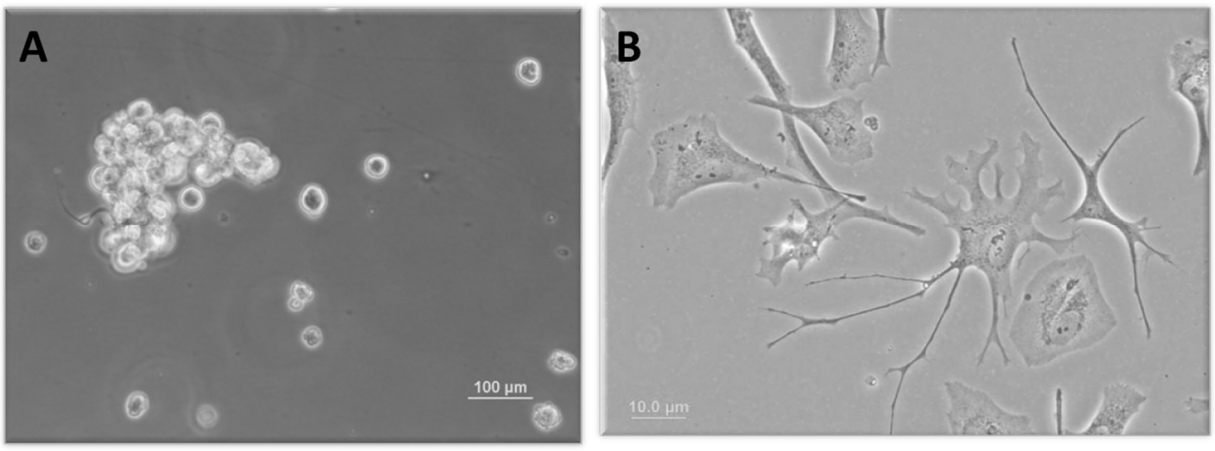

Figure 15. GBM Patient 1 Cells Cultured in Low Attachment Dish and Tissue Culture Treated Dish after being isolated for CD133 in HFB for seven days.

A) GBM Patient 1 cells cultured in a low attachment dish after being isolated for CD133 using the HFB. Picture taken after three days in culture. B) GBM Patient 1 cells cultured in a tissue culture treated dish after isolated for CD133 using the HFB. Picture taken after three days in culture.

To perform the chemotherapy sensitivity assay, a comparable number ($1x10^5$) of bulk of tumor cells grown, as a 2D monolayer, or isolated for CD133 using the HFB (50), were separately plated into 96 wells plates (n-4 replicas) and were treated for an hour with a series of anticancer drugs at a range of concentrations including the clinically relevant dosage (Table 4). Chemotherapy sensitivity assay was performed using a panel of drugs comprising of Temodar, Carboplatin, Cisplatin, Etoposide, Methotrexate, Arabinoside-C, Oxaliplatin, Irinotecan, and Bevacizumab. Sensitivity to chemotherapy was assessed at 24 hours by MTT viability assay. Results were categorized as follows based on the percentage of non-viable cells: responsive (100–60% cell kill), intermediate (30–60% cell kill), and non-responsive (0–30% cell kill). The MTT assay was conducted three separate times with n-4 well replicas/drug/dose each time (Figure 16).

	TMZ	VCR	CPL	CDDP	VP16	MTX	Ara-C	OXA	CPT-11	Avastin
Clinical Dose	150 mg/m²	2 mg/m²	400 mg/m²	80 mg/m²	200 mg/m²	500 mg/m²	2000 mg/m²	80 mg/m²	125 mg/m²	10 mg/kg
Calculated *in vitro* dose	1.23mM	3.5μM	1.72mM	0.43mM	0.54mM	1.36mM	13.16mM	0.32mM	0.32mM	4.4μM

Table 4. Clinical Dose and Calculated *in vitro* Doses of Chemotherapy for GBM Patients.

Chemotherapy sensitivity assay showed that the bulk of tumor of Patient 1 cells grown in monolayer and were intermediately responsive to clinically relevant doses of Temodar (TMZ), Irinotecan (CPT-11) + Bevacizumab (Avastin) combination, Cisplatin (CDDP), Irinotecan (CPT-11), Methotrexate (MTX), and Oxaliplatin (OXA). Interestingly, the CSLCs were only intermediately responsive to a combination of Irinotecan (CPT-11) and Bevacizumab (Avastin) ($p< 0.05$), and were not responsive to any other treatment including Temodar (TMZ) (Figure 16).

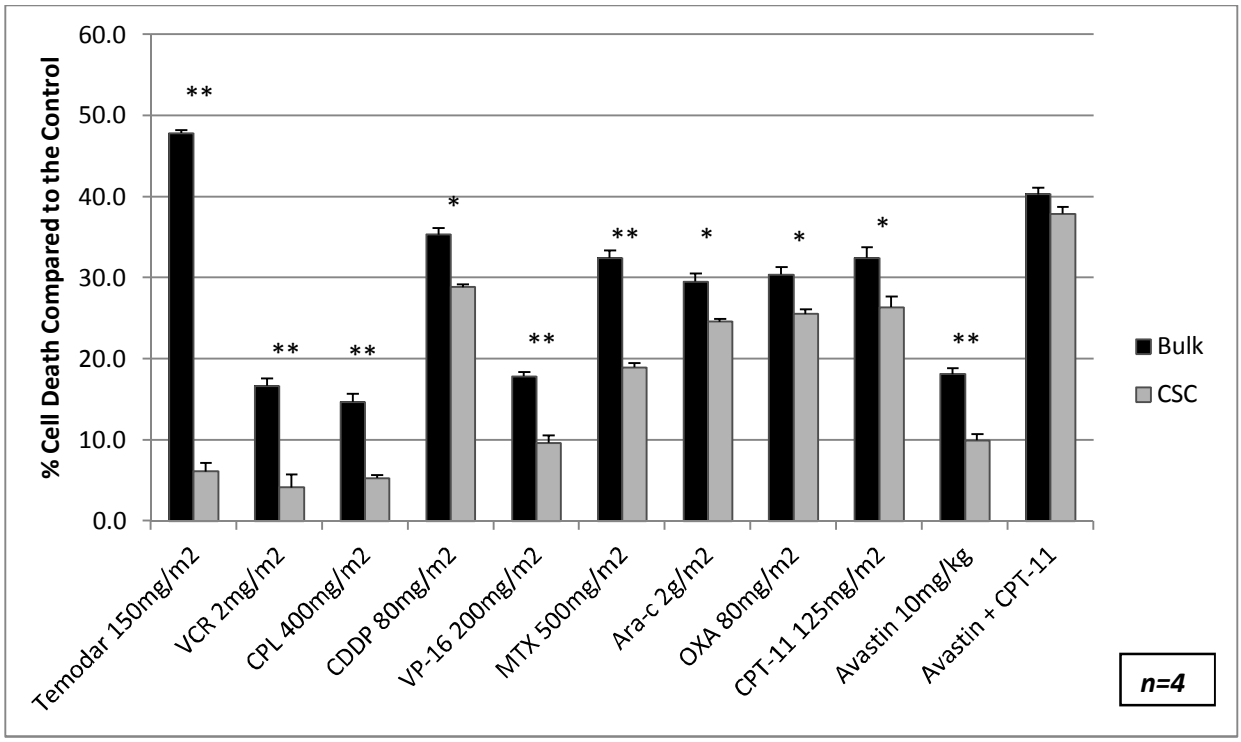

Figure 16. Diagram of Chemotherapy Sensitivity Assay to Assess the Response to GBM Patient 1 cells and CSLCs Using MTT Assay.

1x10³ bulk of tumor cells or CSLCs plated in four replicas into 96-well plates and allowed overnight to attach. Then cells were challenged with a one-hour pulse of varying chemotherapeutic agents. A MTT assay was performed 24 hours following chemotherapy treatments to assess cell viability. Data is plotted in bar graph as responsive (100–60% cell death), intermediately responsive (60–30% cell death), and non-responsive (0–30% cell death). Light grey bars represent sensitivity of CSLCs to chemotherapy with respect to negative untreated control cells. The black bars represent sensitivity of bulk of tumor cells to chemotherapy with respect to negative untreated control cells. Anticancer drugs tested indicated at the bottom of the diagram. Statistical analysis of the significance of the results was performed with a one-way ANOVA. Single asterisk indicate p values of less than 0.05. Double asterisks indicate p values of less than 0.01.

This patient was treated with surgery followed by standard-of-care first-line therapy of Temodar and radiation. At the six months follow-up this patient showed an initial response demonstrated by the diminished size of the tumor at the MRI, but was found to have a tumor relapse at the 12 months follow-up (Figure 17 A, B, & C). Unfortunately, as shown in the follow-up MRI scan at 18 months, the tumor had continued to progress causing the patient to expire (Figure 17 A & D).

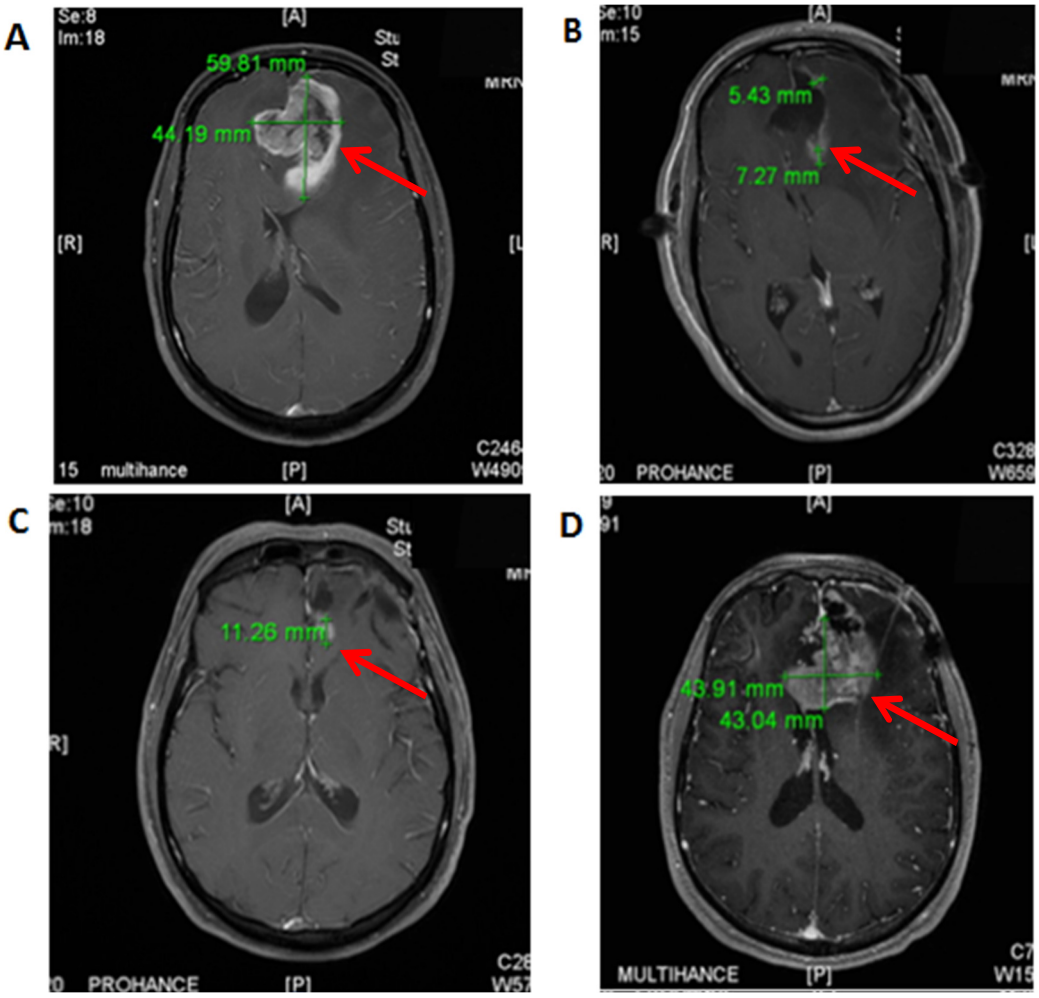

Figure 17. MRI Images from GBM Patient 1.

A. Preoperative MRI of the brain with left frontal lobe GBM crossing the midline into the left frontal lobe & invading the corpus callosum with large amount of associated edema & hydrocephalus. B. Post-operative MRI six months post resection & radiation showing regression of the disease. C. 12 months post therapy MRI showing progression of residual disease. D. 18 months post therapy MRI showing progression of the disease back to bi-frontal mass with increased invasion of corpus callosum and mass affecting on the ventricular system with return of hydrocephalus.

Prediction BULK OF TUMOR	Prediction CSLC	Patient Outcome
Intermediate Response 48.5%±3 cell kill	No Response <10%cell kill	Partial Response

Table 5. GBM Patient 1 Predicted Response and Disease Outcome.

Patient 2

Patient 2 is a 35-year-old male who had a craniotomy on June 11th 2012. Pathology report came back as Glioblastoma IDH-1 wild type. Appropriate informed consent was signed and at the time of the debulking surgery, a sterile biopsy was taken to assess the sensitivity of the tumor cells (bulk of tumor and CSLCs) toward standard-of-care chemotherapy drugs using our chemotherapy sensitivity assay. The biopsy was placed in collection/transport media and tissue was dissociated in the laboratory into a single-cell suspension with the use of a GentleMACS tissue disassociator (Miltenyi, Aubourn, CA). The single-cell GBM suspension was plated as previously reported (49) and cells were cultured as a monolayer for 15 days. Cells were immunophenotyped by flow cytometry using antibodies against CD133, CD34, CD38, CD44, CD24, CXCR4, OCT3/4, and Nanog (Figure 18).

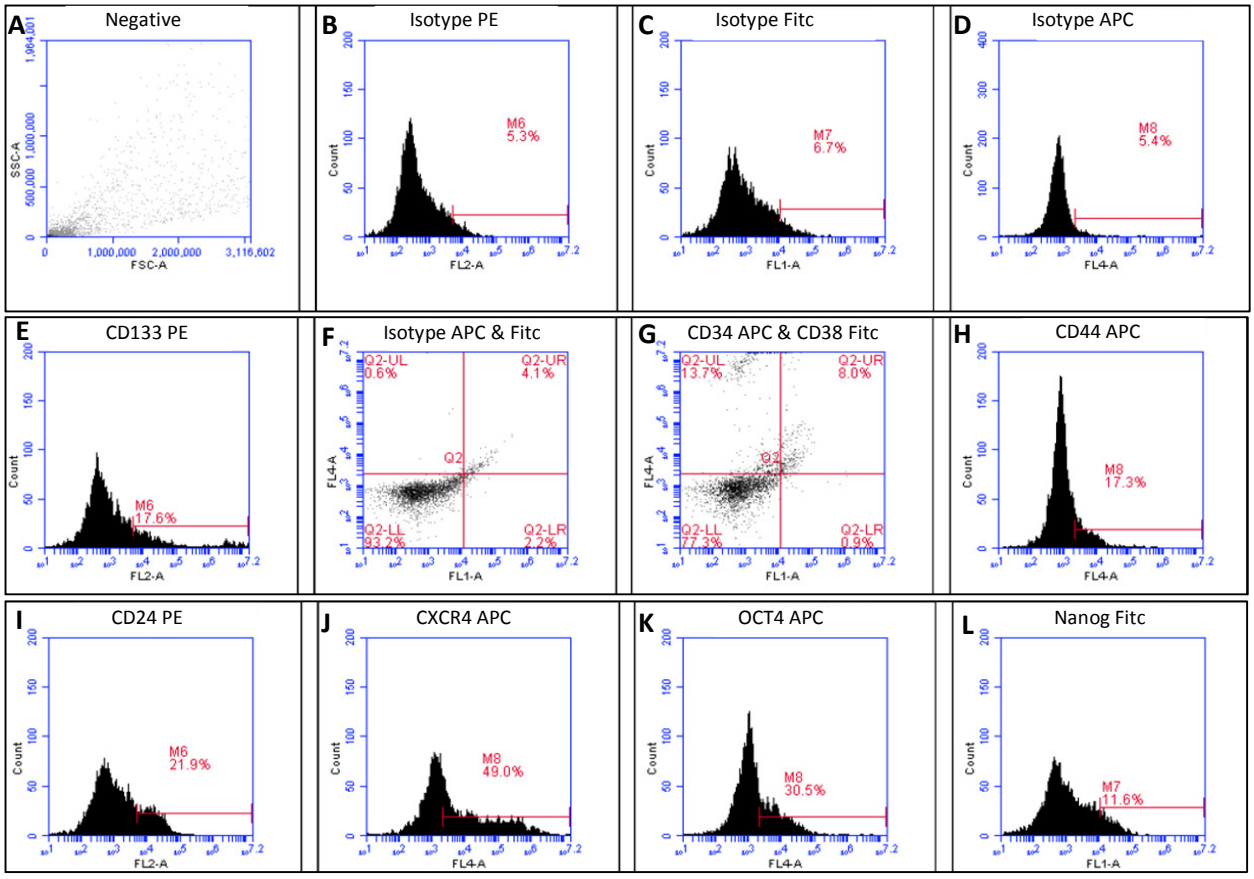

Figure 18. Flow Cytometer Data for GBM Patient 2.

FL1-A is the filter used to measure Fitc, FL2-A is the filter used to measure PE, FL4- A is used to measure APC. A) Negative control showing FSC and SSC on cells not labeled with any antibody. B) Isotype control for PE. C) Isotype control for Fitc. D) Isotype control for APC. E) CD133/2-PE labeled cells. F) Isotype control for Fitc vs APC. G) CD38-Fitc Antibody vs CD34-APC. Upper left quadrate is CD34+/CD38-, upper right quadrant is CD34+/CD38+, lower left quadrant is CD34-/CD38-, and lower right quadrant is CD34-/CD38+. H) CD44-APC labeled cells. I) CD24-PE labeled cells. J) CXCR4-APC labeled cells. K) OCT 3/4-APC labeled cells. L) Nanog-Fitc labeled cells.

	CD133	CD44	CD24	CXCR4	OCT3/4	Nanog	CD34+/CD38-
Percent	12.3	11.9	16.6	43.6	25.1	4.9	13.1

Table 6. Flow Cytometer Data on GBM Patient 2.
Shows the percent of cells tested that are positive for each biomarker compared to the Isotype controls.

The GBM cells were found positive to CD133 (12.3%), CD44 (11.9%), CD 24 (16.6%), CXCR4 (43.6%), OCT3/4 (25.1%), and Nanog (4.9%) when compared to an isotype control antibody (Table 6). A double staining of CD34 and CD38 showed the presence of 1.6% of the cells CD34+/CD38+, and 13.1% CD34+/CD38- cells (Table 6). To expand the CSLC population of CD133+ cells from the GBM primary culture, the cells were cultured as previously described (50). The GBM cells cultured and isolated for CD133 using the HFB for chemotherapy sensitivity assay. When placed directly in non-attaching dish they maintained a cluster assembly (Figure 19A) and would repopulate the heterogeneous GBM culture after ten days in a regular tissue culture dish (Figure 19B).

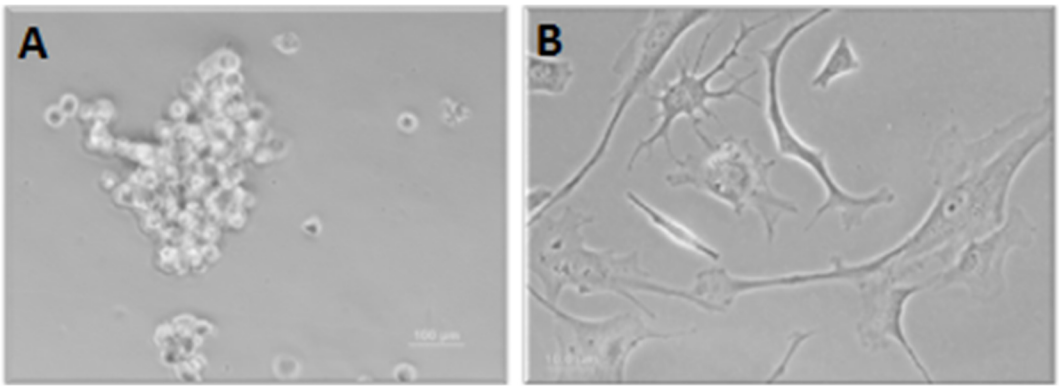

Figure 19. GBM Patient 2 Cells Cultured in Low Attachment Dish and Tissue Culture Treated Dish after being isolated for CD133 using the HFB.

A) GBM Patient 2 cells cultured in a low attachment dish after being isolated for CD133 using the HFB. Picture taken after three days in culture. B) GBM Patient 2 cells cultured in a tissue culture treated dish after being isolated for CD133 using the HFB. Picture taken after three days in culture.

To perform the chemotherapy sensitivity assay $1x10^5$cells of either bulk of tumor cells grown as a 2D monolayer or isolated for CD133 using the HFB (50) were separately plated into 96 wells plates (n-4 replicas) and were treated for an hour with a series of anticancer drugs at a range of concentrations including the clinically relevant dosage as done for GBM Patient 1 (Table 4). Again, chemotherapy sensitivity assay was performed using a panel of drugs comprising of Temodar, Carboplatin, Cisplatin, Etoposide, Methotrexate, Arabinoside-C, Oxaliplatin, Irinotecan, and Bevacizumab. Sensitivity to chemotherapy was assessed at 24 hours by MTT viability assay. Results were categorized as follows based on the percentage of non-viable cells: responsive (100–60% cell kill), intermediate (30–60% cell kill), and non-responsive (0–30% cell kill). The MTT assay was conducted three separate times with n-4 well replicas/drug/dose each time (Figure 20).

This patient was treated with surgery followed by standard-of-care first line chemotherapy with Temodar and radiation therapy. Notably, at the six month post treatment follow-up, the patient showed a positive response with tumor regression as demonstrated by an MRI scan (Figure 21 A, B, & C). A follow-up MRI scan at 18 months showed no evidence of tumor recurrence or progression at the operative bed or distantly (Figure 21 D).

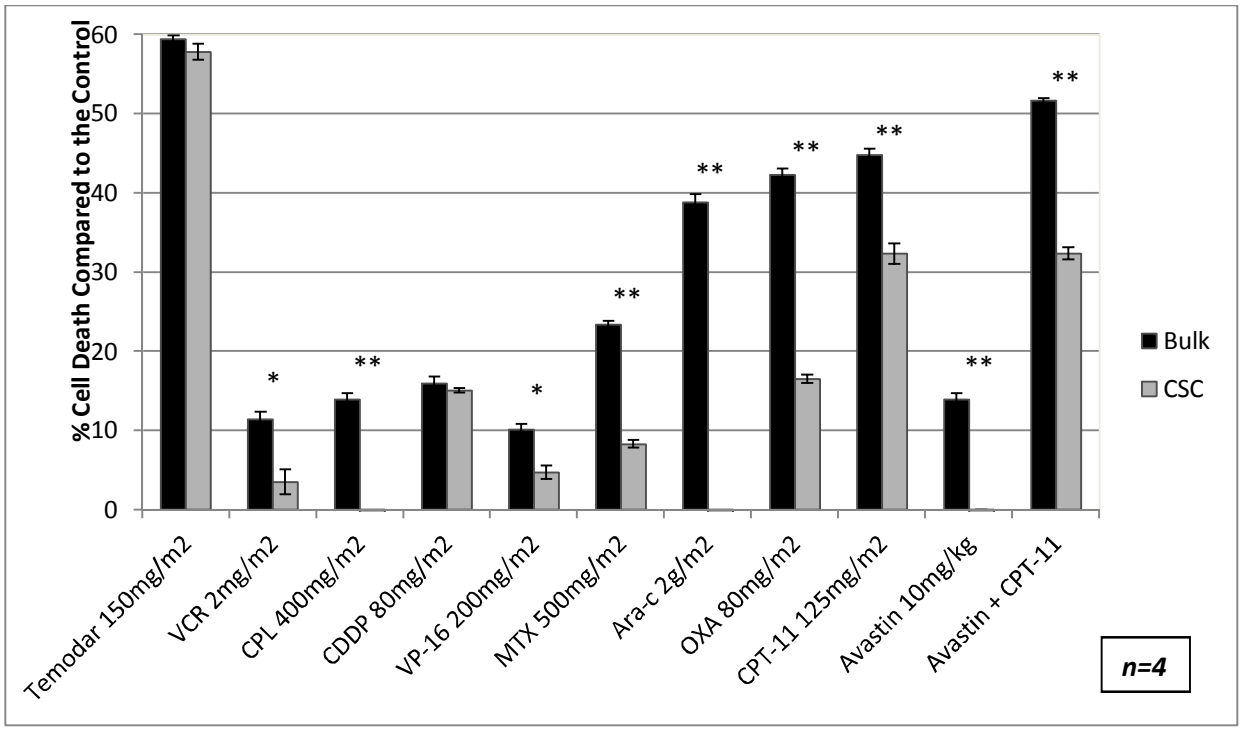

Figure 20. Diagram of Chemotherapy Sensitivity Assay to Assess the Response to GBM Patient 2 cells and CSLCs Using MTT Assay.

1x10³ bulk of tumor cells or CSLCs plated in four replicas into 96-well plates and allowed overnight to attach. Then cells were challenged with a one-hour pulse of varying chemotherapeutic agents. A MTT assay was performed 24 hours following chemotherapy treatments to assess cell viability. Data is plotted in bar graph as responsive (100–60% cell death), intermediately responsive (60–30% cell death), and non-responsive (0–30% cell death). Light grey bars represent sensitivity of CSLCs to chemotherapy with respect to negative untreated control cells. The black bars represent sensitivity of bulk of tumor cells to chemotherapy with respect to negative untreated control cells. Anticancer drugs tested indicated at the bottom of the diagram. Statistical analysis of the significance of the results was performed with a 1-way ANOVA. Single asterisks indicate p values of less than 0.05. Double asterisks indicate p values of less than 0.01.

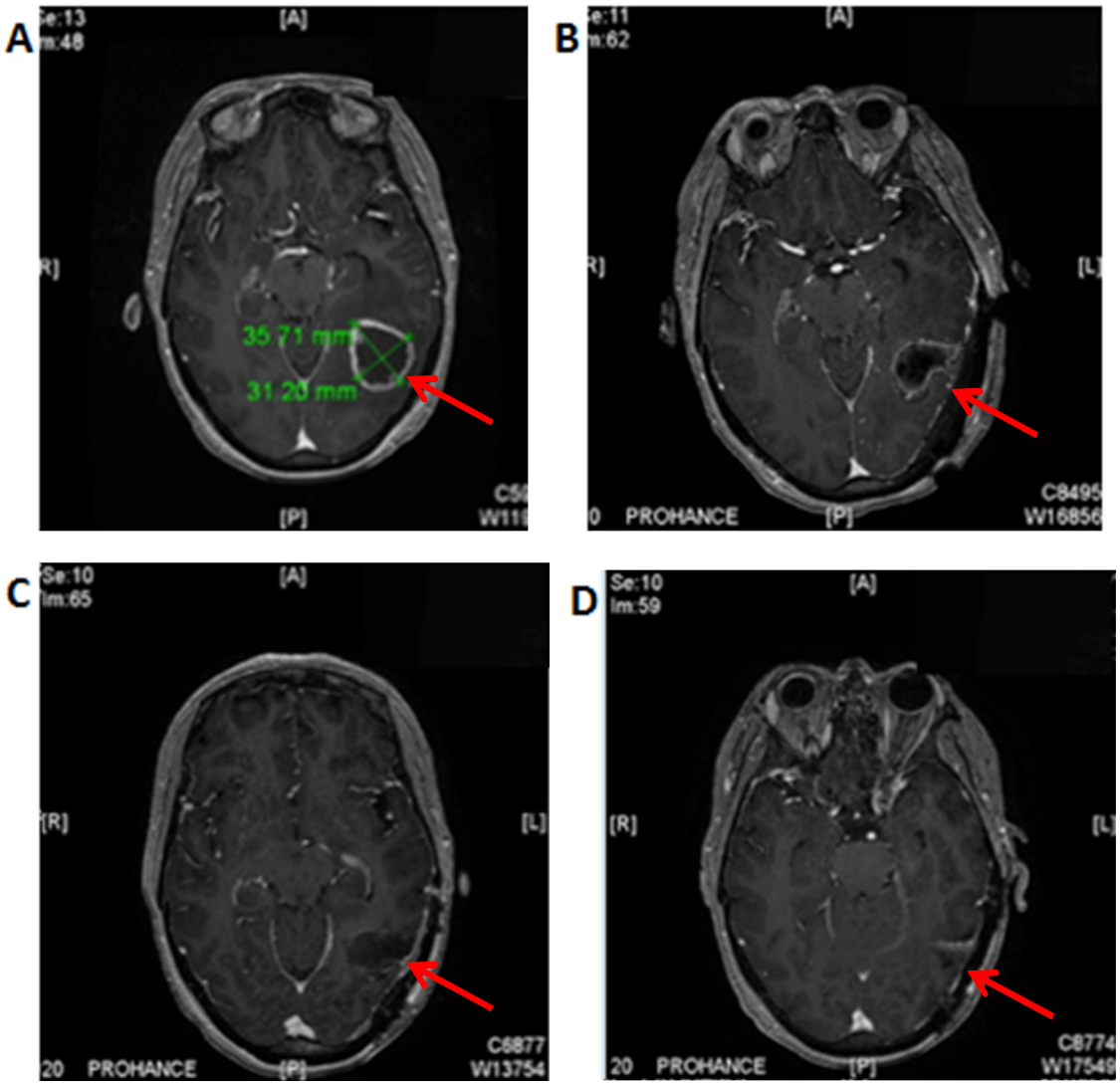

Figure 21. MRI Images from GBM Patient 2.

A. Pre-operative MRI of the brain showing a rim-enhancing GBM in the left cerebrum with edema causing mass effect on the ventricles and ballooning of the left temporal horn & mass effect on the basal cistern. B. Post-surgical resection MRI shows linear non-nodular enhancement likely related to surgery. C. Six months post therapy MRI without evidence of tumor recurrence. D. 18 months post therapy without evidence of tumor recurrence or progression at the operative bed or distantly.

Prediction BULK OF TUMOR	Prediction CSLC	Patient Outcome
Response 60%±1 cell kill	Response 59.4%±3 cell kill	Response

Table 7. GBM Patient 2 Predicted Response and Disease Outcome.

Animal Study

The efficacy of chemotherapies screened *in vitro* by the chemotherapy sensitivity assay was tested on the GBM cells of Patient 1 and Patient 2 that were xenografted in a NOD-Scid mouse model (Figure 22 & 23). Ten athymic NOD-Scid mice were injected in the flank with $1x10^6$ GBM cells mixed to 100 μL of matrigel (BD Biosciences, San Jose, CA) and tumors were grown for ten weeks or until 100 mm^3. Randomized mice were treated by weekly intraperitoneal (i.p.) injections of the different treatments for four weeks and were observed for four more weeks. Group #1 serving as a control received i.p. sterile saline injections. Groups #2–6 were the experimental groups, which received i.p. injections of the least effective chemotherapy, or the most effective, the second most effective, and the most effective combinatorial chemotherapy, as determined by the *in vitro* chemotherapy sensitivity assay.

The tumor xenografts in the Scid mice injected with the least effective chemotherapy as determined by the *in vitro* chemotherapy sensitivity assay grew faster than any other treatment other than the saline control injected mice (Figure 22 Top 23 Top).

Interestingly, tumor regression was initially seen in Scid mice injected with cells from GBM Patient 1 treated TMZ, but after treatment was stopped, at week six, tumor began to grow again (Figure 22 Top). This was confirmed with clinical patient data (Figure 17) that showed after treatment of TMZ and radiation the patient relapsed. The best response by cells from GBM Patient 1 was to combinatorial chemotherapy of CPT-11 + Avastin as determined by the *in vitro* chemotherapy sensitivity assay. Mice weights were measured weekly (Figure 22 Bottom).

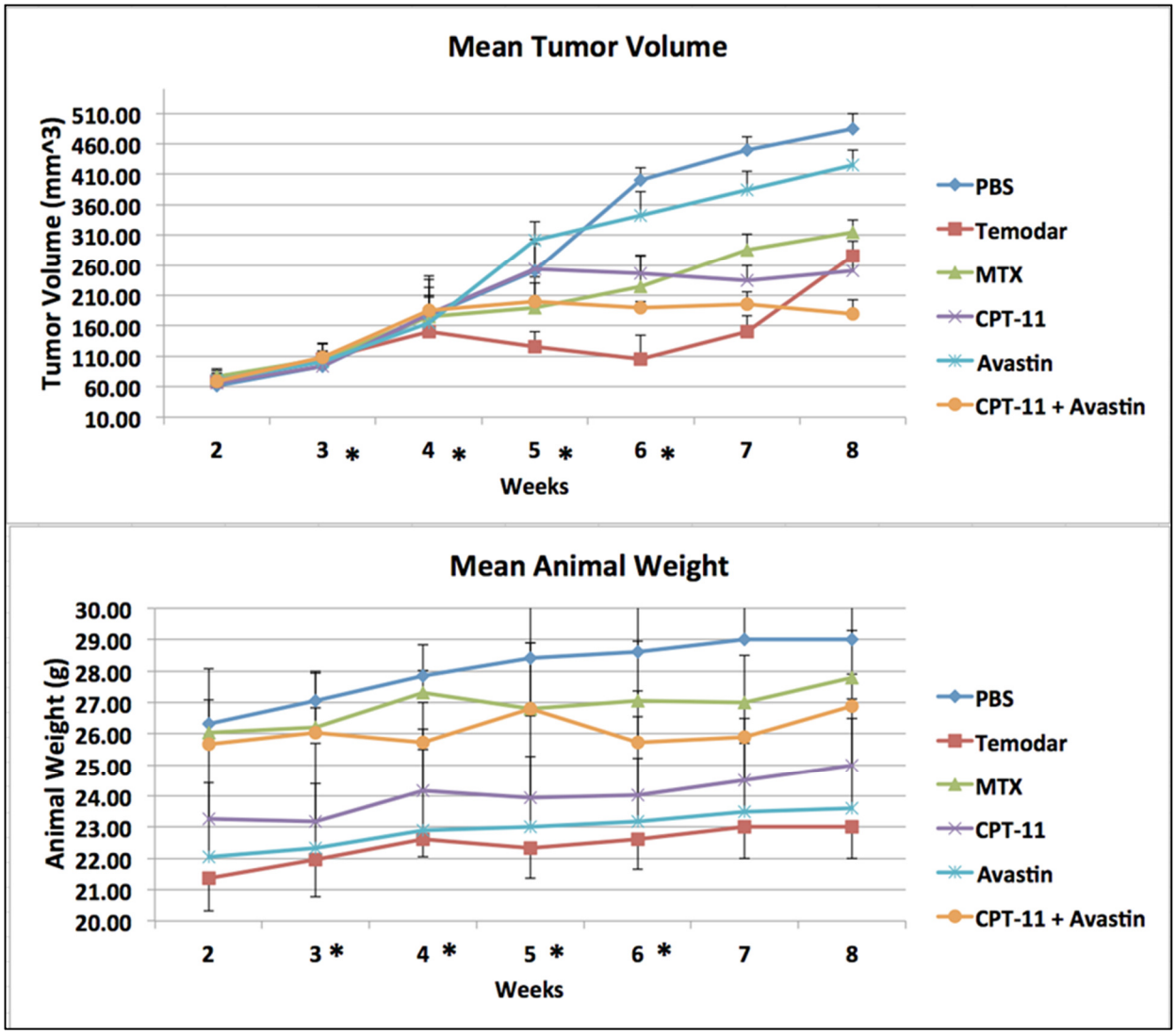

Figure 22. Mean Tumor Volume and Mean Tumor Weight of GBM Patient 1 Derived Xenografts Treated with i.p. Injection of Anticancer Drugs.

Top) Line diagram of the mean volumes in mm³ (±SD) from week 2–8 of ten GBM Patient 1 derived xenografted tumors in NOD-Scid mice following four weeks of treatment with various anticancer drugs. The mean tumor volumes are indicated on the ordinate. Asterisks indicate weeks in which treatment was performed. On the right are indicated the different treatment arms. PBS: saline solution, negative control. Temodar; MTX (Methotrexate), CPT-11 (Irinotecan); Avastin (Bevacizumab); CPT-11 (Irinotecan) + Avastin (Bevacizumab). Bottom) Line diagram of the mean weight in grams (±SD) of ten NOD-Scid mice-bearing patient derived xenografted tumors following four weeks of treatment with various anticancer drugs. The mean tumor weights are indicated on the ordinate. Asterisks indicate weeks in which treatment was performed. On the right are indicated the different treatment arms. PBS: saline solution, negative control. Temodar; MTX (Methotrexate), CPT-11 (Irinotecan); Avastin (Bevacizumab); CPT-11 (Irinotecan) + Avastin (Bevacizumab).

Tumor regression was reported in Scid mice injected with GBM cells from Patient 2, which were treated with the most effective chemotherapy (TMZ) that was confirmed to be the most effective treatment in the *in vitro* chemosensitivity test (Figure 23 Top). This was confirmed by the clinical patient data (Figure 21) that showed after treatment of TMZ and radiation the patient was in remission. The second best response of GBM Patient 2 was to combinatorial chemotherapy of CPT-11 + Avastin as predicted by the *in vitro* chemotherapy sensitivity assay and as determined by the xenograft treatment. Mice weights were measured weekly (Figure 23 Bottom).

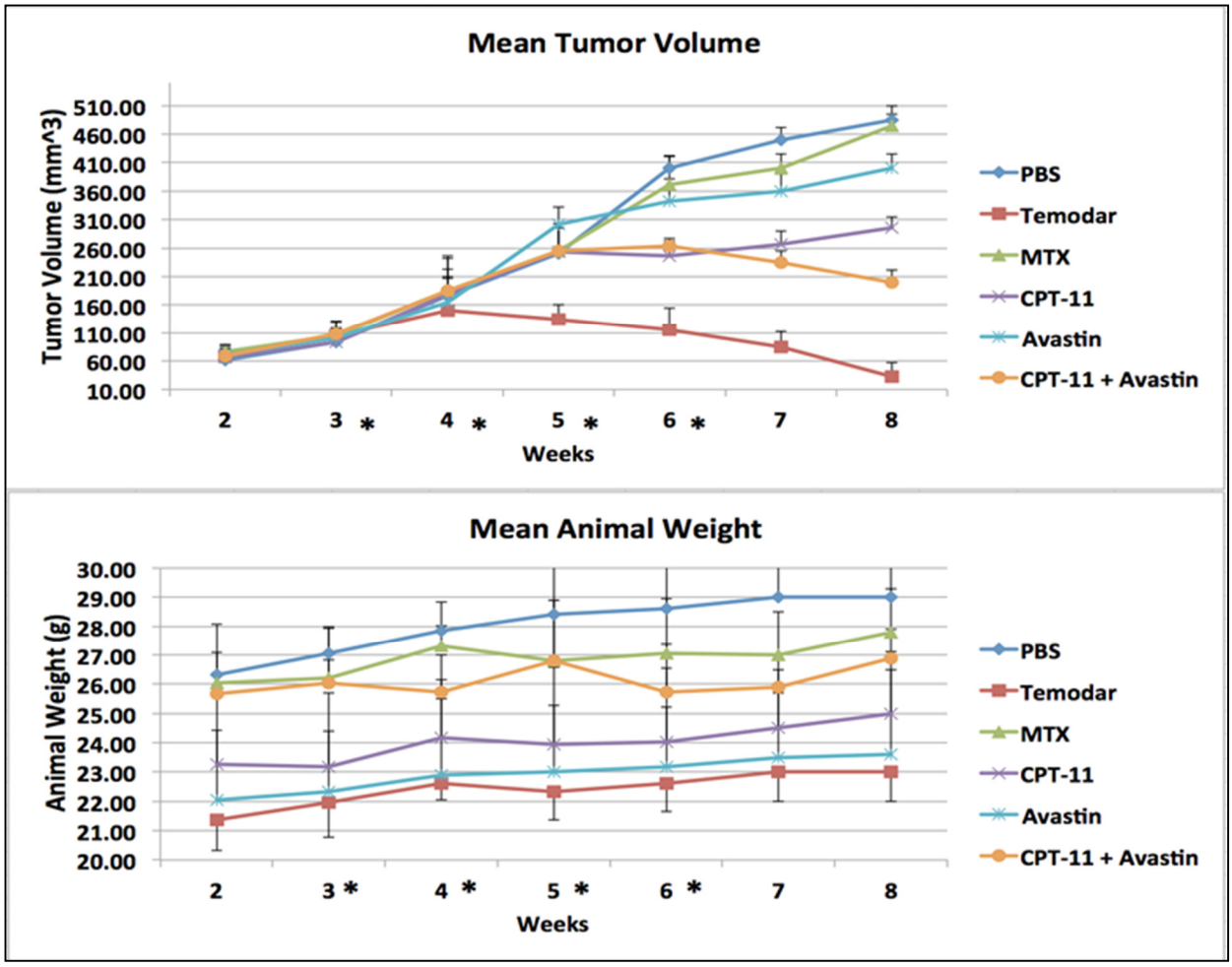

Figure 23. Mean Tumor Volume and Mean Tumor Weight of GBM Patient 2 Derived Xenografts Treated with i.p. Injection of Anticancer Drugs.

Top) Line diagram of the mean volumes in mm³ (±SD) from week 2–8 of ten GBM Patient 2 derived xenografted tumors in NOD-Scid mice following four weeks of treatment with various anticancer drugs. The mean tumor volumes are indicated on the ordinate. Asterisks indicate weeks in which treatment was performed. On the right are indicated the different treatment arms. PBS: saline solution, negative control. Temodar; MTX (Methotrexate), CPT-11 (Irinotecan); Avastin (Bevacizumab); CPT-11 (Irinotecan) + Avastin (Bevacizumab). Bottom) Line diagram of the mean weight in grams (±SD) of ten NOD-Scid mice-bearing patient derived xenografted tumors following four weeks of treatment with various anticancer drugs. The mean tumor weights are indicated on the ordinate. Asterisks indicate weeks in which treatment was performed. On the right are indicated the different treatment arms. PBS: saline solution, negative control. Temodar; MTX (Methotrexate), CPT-11 (Irinotecan); Avastin (Bevacizumab); CPT-11 (Irinotecan) + Avastin (Bevacizumab).

DISCUSSION

Treatment for Glioblastoma (GBM) is often a combinatorial approach that includes surgery, radiation therapy, and chemotherapy. Temozolomide (TMZ) is the primary chemotherapeutic agent used in the treatment of GBM but is often non-curative and patients frequently experience tumor relapse due to chemoresistance.

As shown in this study of two GBM cases the different responses to chemotherapy determined by the chemosensitivity assay on the CSLCs resulted in a distinct and opposite outcome for the patient.

To advance our ability to more thoroughly understand the CSLC driving force within heterogeneous tumors such as GBMs, a procedure that can successfully enrich the CSLC population from tumor biopsy specimens was developed. These enriched CSLCs are then used to assess their intrinsic resistance or sensitivity to chemotherapy vs. the resistance of primary cultures derived from the total heterogeneous tumor. This method successfully facilitated the enrichment of CD133+ CSLCs from fresh GBM biopsy tissues allowing the selective evaluation of chemotherapy response of the CSLC population compared to the response of the parenteral heterogeneous tumor cell population. Results using this procedure showed that GBM tumors do contain populations of CSLCs that can be selectively enriched, and that the chemoresistance or sensitivity status of CSLCs to TMZ predicted the patients' treatment outcome in GBM patients receiving the standard-of-care Temozolomide (TMZ) regimen.

GBM Patient 1 was diagnosed with a GBM IDH-1 mutant, which should have experienced a better outcome following treatment with TMZ. However, following standard chemoradiation treatment a recurrence was observed within six months. GBM patient 2, who was diagnosed with a GMB, but IDH-1 wild type, should have had worse prognosis following standard chemoradiation treatment. Instead patient 2 showed a positive response with tumor

regression as demonstrated by MRI scans indicating that genetic analysis, of a single marker, to predict tumor response may not be an accurate method of patient stratification.

This study accurately predicted that Patient 1 would relapse because the CSLCs would not be affected by the TMZ treatment, thereby causing the CSLCs to reinitiate the tumor. In contrast, Patient 2 was predicted to respond well to the treatment received by the chemotherapy sensitivity assay. Figure 24 shows the responses of GBM Patient 1 and 2 to TMZ using the chemotherapy sensitivity assay.

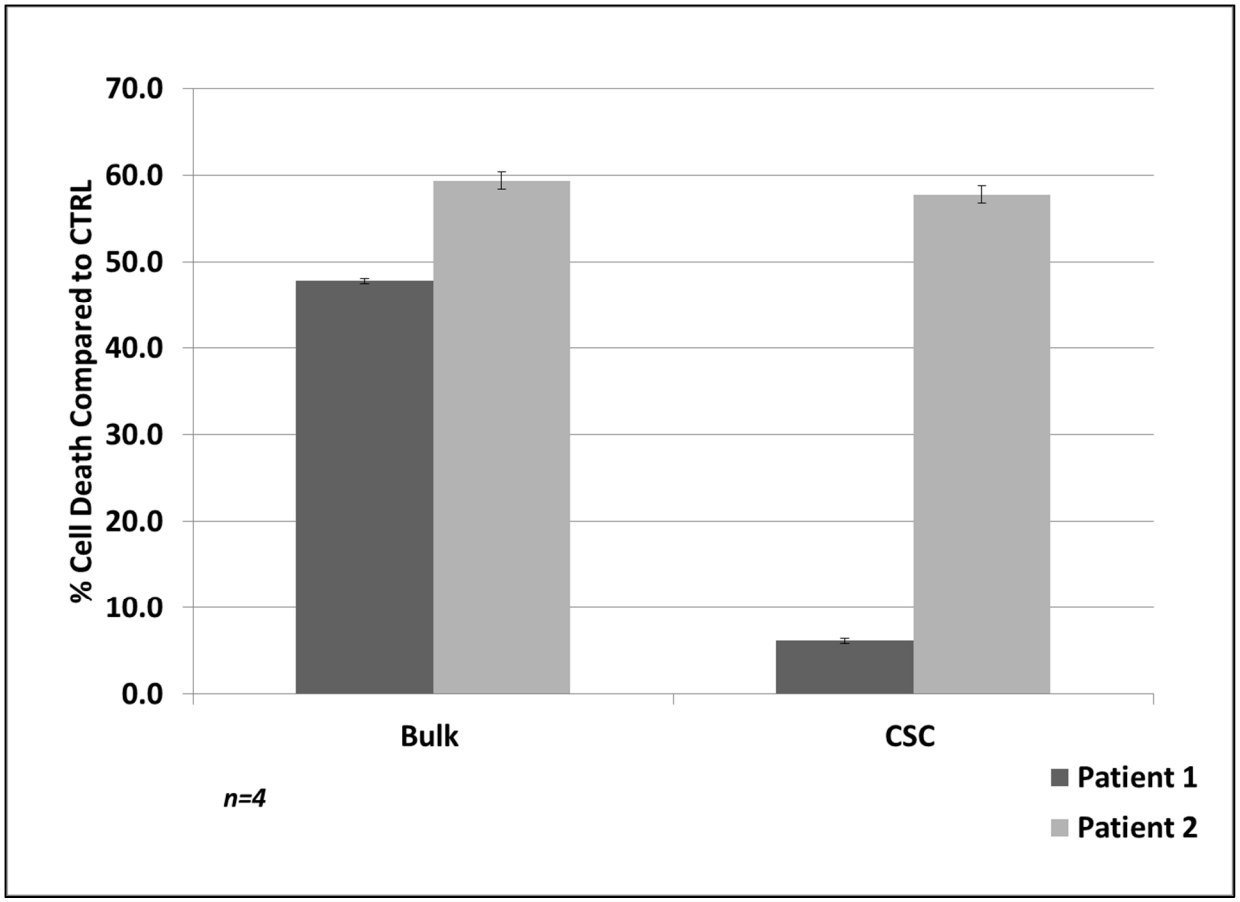

Figure 24. Diagram of GBM Patient 1 and Patient 2 Response to TMZ using the Chemotherapy Sensitivity Assay on both the Bulk of the tumor and the CSLCs.

This outcome was also challenged using NOD-Scid mice xenografts treated with the most effective, the second most effective, and the most effective combinatorial chemotherapy as determined by the *in vitro* chemotherapy sensitivity assay. As expected, results from tumor xenografts generated by injecting enriched GBM CSLCs from the two patients in the flank of *NOD-Scid* mice, followed by treatment with select chemotherapies, were also found to be in agreement with the clinical outcomes for the patients' response to TMZ at follow-up. Regression was observed at first in tumor xenografts from Patient 1 cells treated with TMZ; however, following cessation of the TMZ treatment relapse was observed (Figure 22). Interestingly,

xenografts from Patient 2 treated with TMZ, responded well to the treatment and a regression of the tumors was observed (Figure 23).

This data shows the importance of determining the sensitivity of CSLCs to chemotherapy and their role in predicting patient tumor response following chemotherapy. This data further supports our belief that long-term tumor response in GBM is in fact more dependent on the intrinsic sensitivity or resistance of the CSLC population than the general tumor cell population and needs to be closely studied to determine how this population can be targeted for therapy. This method will provide critical information about an individual patient's possibility to achieve a complete tumor response status before implementing the patient's treatment plan. Although the chemotherapy sensitivity assay results of these two cases of GBM showed to be clinically relevant, this laboratory is currently conducting a larger study on a series of GBMs to determine the prognostic accuracy of this assay.

CHAPTER 4: CONCLUSIONS AND FUTURE DIRECTIONS

Chapter 1 reviewed the current literature on CNS cancer treatments, cancer stem cell theory, and standard-of-care anticancer drugs. In this chapter the potential for chemotherapy sensitivity assay on cancer stem-like cells to establish personalized patient therapy was highlighted. The method of how this assay is performed and its validation is also outlined in this chapter.

Chapter 2 discussed the relevance and outcome of two patients affected by Ependymoma. The ependymoma of Patient 1 was found sensitive to a combination of Irinotecan + Avastin, but resistant to Oxaliplatin. This data was confirmed by an animal study in which mice treated with Oxaliplatin and failing therapy could be rescued with a combination of Irinotecan + Avastin. This study also showed that an adjuvant supplementation of BITC extracted from broccoli sensitized CSLCs to chemotherapy and increased its efficacy both *in vitro* and *in vivo*. Unfortunately, the ependymoma of patient 2 was found not responding to the chemotherapies used and was also resistant to the same anticancer drugs when tested in the *in vitro* chemosensitivity assay.

In Chapter 3, the chemotherapy sensitivity assay was investigated in two glioblastoma cases. The CSLCs of GBM Patient 1 were found resistant to TMZ by the chemotherapy sensitivity assay. Following a regimen of chemoradiation with TMZ this patient relapsed after a year. The clinical outcome of patient 1 was found in accordance with both the *in vivo* and *in vitro* studies performed in the laboratory. GBM Patient 2 was also treated with chemoradiation with TMZ, but interestingly this patient showed a completely different clinical outcome being free from progression for 18 months. In patient 2, the chemotherapy sensitivity assay accurately

predicted that both bulk of the tumor cells and CSLCs would respond to TMZ, which was also confirmed by a study conducted using mice xenografts.

The chemotherapy sensitivity assay has shown great promise in these four cases. This assay demonstrated that the CSLC population must be assayed to better predict patient outcomes because of the known resistance of CSLCs to anticancer drugs. By combining the information gathered for both Bulk and CSLC population, new treatment modalities could be established that could lead to increased survival rates.

In addition, the clinical study presented above suggests that future studies of natural compounds such as BITC and others should be performed to further increase our understanding of the potential of botanical extracts and compounds as adjuvant to chemotherapy.

One of the shortcomings of the present study is that O 6-methylguanine DNA methyltransferase (MGMT) status was not investigated on the samples. MGMT gene encodes for a protein that is involved in DNA repair by removing the alkyl groups (108). This is problematic for proscribed chemotherapeutic drugs, like TMZ, that are alkylating agents that place alkyl groups on the DNA so that replication cannot occur (108). MGMT activity is regulated by epigenetic factors that methylate the promoter region of the gene to reduce the amount of MGMT in a GBM cell (108). MGMT gene methylation has been shown to be associated with improved outcome in GBM and may be a predictive marker of sensitivity to alkylating agents. While MGMT status of these tissues has not been examined, it is an experiment to consider for the future.

Another shortcoming of the present studies is the lack of large clinical data; however, the clinical data from a series of twenty-five GBMs is currently being evaluated. In conclusion, this

present body of work supports the knowledge that chemotherapy sensitivity assays on CSLCs are a promising tool to more accurately predict patient outcome following anticancer treatment. This assay has the potential to be standardized and used in clinical settings, being novel in its approach to analyze the sensitivity/resistance of CSLCs to anticancer drugs and hopefully sparing future patients from being treated with ineffective drugs.

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APPENDIX

Office of Research Integrity IRB Approval

MARSHALL UNIVERSITY
www.marshall.edu

Office of Research Integrity
Institutional Review Board
401 11th St., Suite 1300
Huntington, WV 25701

FWA 00002704

IRB1 #00002205
IRB2 #00003206

April 16, 2012

Pier Paolo Claudio, MD, PhD
Marshall University, Department of Biochemistry and Microbiology

RE: IRBNet ID# 326290-1
At: Marshall University Institutional Review Board #1 (Medical)

Dear Dr. Claudio:

Protocol Title:	[326290-1] Chemotherapy Resistance and Sensitivity Testing in Brain and Spine Tumors	
Expiration Date:	April 16, 2013	
Site Location:	MU,CHH	
Submission Type:	New Project	APPROVED
Review Type:	Expedited Review	

In accordance with 45CFR46.110(a)(5), the above study was granted Expedited approval today by the Marshall University Institutional Review Board #1 (Medical) Chair for the period of 12 months. The approval will expire April 16, 2013. A continuing review request for this study must be submitted no later than 30 days prior to the expiration date.

If you have any questions, please contact the Marshall University Institutional Review Board #1 (Medical) Coordinator Trula Stanley, MA, CIC at (304) 696-7320 or stanley@marshall.edu. Please include your study title and reference number in all correspondence with this office.

MARSHALL UNIVERSITY
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Animal Resource Facility

DATE:	October 3, 2012
TO:	Pier Paolo Claudio
FROM:	Marshall University IACUC
IACUC #:	519
PROJECT TITLE:	[360347-6] Pre-clinical evaluation of chemotherapy treatment
SUBMISSION TYPE:	Revision
ACTION:	APPROVED
APPROVAL DATE:	10/3/2012
EXPIRATION DATE:	7/14/2015
REVIEW TYPE:	

Thank you for your submission of Revision materials for this research project. The Marshall University IACUC has APPROVED your submission. All research must be conducted in accordance with this approved submission.

This submission has received Full Committee Approval.

Please note that any revision to previously approved materials must be approved by this committee prior to initiation. Please use the appropriate revision forms for this procedure.

Please report all NON-COMPLIANCE issues regarding this project to this committee.

This project requires Continuing Review by this office on an annual basis. Please use the appropriate renewal forms for this procedure.

If you have any questions, please contact Monica Valentovic at (304) 696-7332 or valentov@marshall.edu. Please include your project title and reference number in all correspondence with this committee.

Monica A. Valentovic, Ph.D.
Chairperson, IACUC

Curriculum Vitae

Sarah Elizabeth Daron-Mathis

1141 10th Ave, Huntington, WV 25701
Home 304-730-5456 • s.daronmathis@gmail.com

Present Positions
PhD Graduate student and researcher, Biomedical Sciences, School of Medicine, Marshall University, Huntington, WV 2009-Present

Education
PhD Biomedical Sciences, Marshall University, Huntington, WV May 2015
MS Biological Sciences, Marshall University, Huntington, WV May 2009
BS Biological Sciences, Marshall University, Huntington, WV July 2007

Teaching Experience
Adjunct Professor, Microbiology GE 257, ITT Tech, 2014
Lab Instructor, Cell Biology BSC 322, Marshall University, 2009
Lab Instructor, Cell Biology BSC 322, Marshall University, 2008
Substitute Lecturer, Human Biology BSC 105, Marshall University, 2008
Lab Instructor, Principles of Biology, Clearwater Christian College, 2003

Research Laboratory Experience
Research Assistant, Biochemistry and Microbiology Department, Marshall University (2009-present)
International Exchange Graduate Research, Council for National Research (CNR) Rome, Italy (Spring 2009)
Research Assistant, Biology Department, Marshall University (2006-2009)

Qualification Summary & Skill Set
Biomedical Lab Manager with expertise in microbiology techniques, including aseptic technique, cell culture, autoclaving and media preparation. Experienced in tissue engineering and chemo-sensitivity assays.

- Expert in Cell Culture of established and Primary Cell lines
- Experienced in tissue engineering
- Expert in Microgravity engineering
- Proficient in analytical techniques
- Expert in Flow Cytometry
- Skilled in viability assays
- Expert in Microscopy
- Operations management

- Staff supervision and training
- Complex problem solving
- Quality control
- Adept in creation of SOP

Research Grants
Graduate Research Fellowship Program - NASA West Virginia Space Grant Consortium (June 2008)
Charles C. Gould Scholarship- Marshall University (May 2008)
Travel Grant - American Society for Gravitational and Space Biology (October 2007)
Travel Grant - NASA West Virginia Space Grant Consortium (September 2007)
Graduate Research Fellowship Program - NASA West Virginia Space Grant Consortium (May 2007)
NASA Space Grant Scholarship – NASA (November 2006)
College of Science Tuition Waiver Scholarship Award - Marshall University (November 2006)

Posters
Undergraduate Research Day at the Capitol February 8th 2007
Presentation was given at West Virginia's state capital to the states delegates about the current research project on "Bioreactor Production of Plant Pharmaceuticals."

Sigma Xi April 28th 2007
Presentation was given at Marshall University to fellow students and faculty about the current research project on "Medicinal Plant Cell Cultures in Microgravity-based Bioreactor."

STaR Symposium September 17th 2007
Presentation was given at Waterfront Place Hotel in Morgantown, WV to fellow students and faculty in the state of West Virginia about the current research project on "Production of Bioproducts in a Microgravity-based Bioreactor."

American Society for Gravitational Space Biology 23rd Annual Meeting
October 25-28th 2007
Presentation was given at AMES Research Center to fellow students and faculty from around the world about the current research project on "Selection of Tumor Stem Cells from Tumor Cell lines."

Life in Space for Life on Earth June 22-27th 2008
This presentation was given at a conference in Angers, France. This conference was hosted by European Space Agency (ESA) along with International Society for Gravitational Physiology (ISGP), American Society for Gravitational Space Biology (ASGSB), and European Low Gravity Research Association (ELGRA). Presentation was given about "Rapid Selection and Proliferation of Cancer Stem Cells in a NASA Developed Microgravity Bioreactor."

Advances in Cell Differentiation and Development *Symposium* November 14th, 2008

Cell Differentiation and Development Center (*CDDC*) First Annual Symposium was held at Memorial Student Center, Marshall University where a Presentation was given to fellow students and faculty from the region on the "Selection of Tumor Stem Cells from Tumor Cell lines." This was a competitive poster competition where I received first place.

STaR Symposium April 13-14th, 2009

Presentation was given at Charleston Marriott Town Center in Charleston, WV to fellow students and faculty in the state of West Virginia about the current research project on "Rapid Selection and Proliferation of Cancer Stem Cells in a NASA Developed Microgravity Bioreactor: Chemotherapeutic Implications."

AICR Research Conference November 4-6th, 2009

AICR Research Conference held its 20th Annual Conference in Washington, D.C. at the Hilton. Presentations were given to fellow students and faculty from the region on "Omega-3 and -6 fatty acids select proliferate and sensitize colorectal cancer stem-like cells to chemotherapy."

Marshall University Research Day March 20th, 2012

This presentation was given orally at Marshall University Joan C. Edwards School of Medicine hosts 24th annual Research Day was held at Cabell Huntington Hospital in Huntington, WV to fellow students and faculty on "Personalized Chemotherapy Identified for a Case of Progressing Spinal Ependymoma."

Advances in Cell Differentiation and Development *Symposium* March 23rd, 2012

Cell Differentiation and Development Center (*CDDC*) Second Annual Symposium was held at Memorial Student Center, Marshall University where a Presentation was given to fellow students and faculty from the region on the "Personalized Chemotherapy Identified for a Case of Progressing Spinal Ependymoma." This was a competitive poster competition where I received first place in the graduate student category.

STaR Symposium April 20-21st, 2012

Presentation was given at West Virginia State University in Institute, WV to fellow students and faculty in the state of West Virginia about the current research project on "Personalized Chemotherapy Identified for a Case of Progressing Spinal Ependymoma."

Center for Clinical and Translational Science Spring Conference (CCTS) March 29th 2012.

Presentation was given in Lexington Kentucky to fellow students and faculty of the region about the current research project on "Personalized Chemotherapy Identified for a Case of Progressing Spinal Ependymoma."

Appalachian Regional Cell Conference (ARCC) October 12th 2012.

Presentation was given at the Charleston Area Medical Center in Charleston, WV to fellow students and faculty of the region about the current research project on "Personalized Chemotherapy Identified for a Case of Progressing Spinal Ependymoma."

Marshall University Research Day March 11th-12th, 2013

This presentation was given orally at Marshall University Joan C. Edwards School of Medicine hosts 25th annual Research Day was held at Cabell Huntington Hospital in Huntington, WV to fellow students and faculty on "Resveratrol suppresses lipogenesis, growth and viability of pancreatic and breast cancer stem-like cells."

Appalachian Regional Cell Conference (ARCC) October 26th 2013.

Oral presentation was given at the Charleston Area Medical Center in Charleston, WV to fellow students and faculty of the region about the current research project on "ChemoID® a Personalized Chemotherapy Identification for a Case of Progressing Spinal Ependymoma"

Publications

Chemo-Predictive Assay for Targeting Cancer Stem-Like Cells in Patients Affected by Brain Tumors. Sarah E. Mathis, Anthony Alberico, Rounak Nande, Walter Neto, Logan Lawrence, Danielle R. McCallister, James Denvir, Gerrit A. Kimmey, Mark Mogul, Gerard Oakley, Krista L. Denning, Thomas Dougherty, Jagan V. Valluri, Pier Paolo Claudio. PLoS ONE, 2014 Aug 21; 9(8): e105710.

Rapid Selection and Proliferation of CD133 (+) Cells from Cancer Cell Lines: Chemotherapeutic Implications. Sarah E. Kelly, Altomare Di Benedetto, Adelaide Greco, Candace M. Howard, Vincent E. Sollars, Donald A. Primerano, Jagan V. Valluri, Pier Paolo Claudio. PLoS ONE, 2010 Apr 8;5(4):e10035

Eradication of therapy-resistant human prostate tumors using an ultrasound-guided site-specific cancer terminator virus delivery approach. Greco A, Di Benedetto A, Howard CM, Kelly S, Nande R, Dementieva Y, Miranda M, Brunetti A, Salvatore M, Claudio L, Sarkar D, Dent P, Curiel DT, Fisher PB, Claudio PP. Mol Ther. 2010 Feb;18(2):295-306.

Protein Expression in Glycine max Cells under Microgravity Conditions. Jagan Valluri, W. Kelly, and S. Kelly October 2008 Gravitational and Space Biology

Honor and Awards

First Place poster competition for graduates and post docs –Advances in Cell Differentiation and Development Symposium	March 2012
Best in Group poster competition - ARCC Applachain Regional Cell Conference	October 2012
First Place poster competition for graduates and post docs -Advances in Cell Differentiation and Development Symposium	November 2008
Graduate Research Fellowship Program - NASA West Virginia Space Grant Consortium	June 2008
Charles C. Gould Scholarship- Marshall University	May 2008
Travel Grant - American Society for Gravitational and Space Biology	October 2007
Travel Grant - NASA West Virginia Space Grant Consortium	September 2007
Graduate Research Fellowship Program	May 2007

- NASA West Virginia Space Grant Consortium

NASA Space Grant Scholarship - NASA	November 2006
College of Science Tuition Waiver Scholarship Award - Marshall University	November 2006
Dean's List - Marshall University	May 2006
National Dean's List - The National Dean's List	May 2006
Dean's List - Marshall University	December 2005
Dean's List - University of Cincinnati	December 2003
Dean's List - Clearwater Christian College	May 2003

Associations

National Association of Professional Women (NAPW) 2012-Present, Member
International Bujinkan Dojo Association (IBDA) 2011-Present, Member
Bujinkan Student Organization 2011-Present, Secretary (2011-2012) President (2012-2013)
Southside Elementary Parent Teacher Organization (PTO) 2010-Present, Member
American Society for Gravitational Space Biology (ASGSB) 2007-2008, Student Member
Graduate Student Organization (GSO) 2009-Present, Student Member

Volunteer work

Cabell Huntington Hospital, Physical Therapy Department May 2006- August 2006
WV EPsCOR Both worker at state fair August 2007
Teaching Substitute for Biology 105 Dr. Valluri April 2008
Visiting Research at CNR in Rome, Italy Spring 2009
Team Mom for DC Express peewee football 2010 season
Game Night Organizer for Ona Children's Home March 2011
Guest Lecturer for Biology 105 at ITT tech August 5th 2011