THE EFFECT OF BLOOD-BRAIN BARRIER DISRUPTION ON CANCER-ASSOCIATED BIOMARKER SHEDDING FROM MURINE GLIOMAS

A Thesis
Presented to
The Academic Faculty

by

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In Partial Fulfillment
of the Requirements for the Degree
B.S. in Biomedical Engineering in the
College of Engineering

Georgia Institute of Technology
May 2019

THE EFFECT OF BLOOD-BRAIN BARRIER DISRUPTION ON CANCER-ASSOCIATED BIOMARKER SHEDDING FROM MURINE GLIOMAS

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ABSTRACT

Focused Ultrasound disruption is a novel therapy that could facilitate the use of liquid biopsies for detection and observation of cancers in immune privileged organs. These alternatives to traditional biopsies are used to analyze molecules in circulation which could be less invasive and more effective measures of tumor progression. This study aims to determine whether targeted Focused Ultrasound-induced blood-brain barrier disruption allows for greater concentrations of cancer-associated molecules from malignant brain tumors to enter the circulation. This determination is based on parallel experimentation to detect these molecules both *in vitro* and *in vivo* using genetic sequencing and bioluminescent response from Gaussia Luciferase and observing the effect with and without Focused Ultrasound therapy. Polymerase chain reaction was used to amplify Gaussia Luciferase and was found to be sensitive enough to detect a single copy of DNA. While this study presents preliminary results from these experiments, the results of this inquiry have yet to be finalized and future experimentation with be needed.

CHAPTER 1

INTRODUCTION

It is well-known that the protective blood brain barrier (BBB) that surrounds the vessels in the central nervous system (CNS) poses a major obstacle to the transport of polar and large molecules between the interstitial fluid of the brain and blood. The BBB is made up of tight junctions between adjacent endothelial cells that line the blood vessels and restrict the passage of molecules to those under 400 Da. The vessel is then surrounded by pericytes and astrocytes which maintain the structural integrity of the barrier (Burgess, Shah, Hough, & Hynynen, 2015). While the BBB works to protect the delicate brain microenvironment needed for proper neuronal function, it also makes it difficult to both treat ailments of the CNS like brain tumors and monitor the secreted molecules from these tumors (Ballabh, Braun, & Nedergaard, 2004). Indeed, this membrane is cited as the single largest obstacle to drug delivery to the brain (Burgess et al., 2015).

Glioblastoma multiforme (GBM) is a particularly aggressive brain tumor with only 5-10% of patients surviving two years after diagnosis (Szatmári et al., 2006). The traditional treatments for glioblastoma are tumor resection of the mass which, due to the inability to ensure the complete removal via surgery alone, is then followed by radiation. Unfortunately, studies suggest that these invasive procedures have little effect on the survival rate of patients as radioresistance is often a characteristic of GBM. (Szatmári et al., 2006). This assertive cancer in an immune privileged organ serves as motivation to explore new methods of bypassing the BBB. Overcoming the obstacle of the BBB would allow for more effective and less invasive therapies to reach glioblastomas.

Some of the current methods that are used to bypass the BBB include direct injections to the brain, intrathecal injections to the cerebrospinal fluid, and intranasal delivery. The

former two methods are complicated by the risk of hemorrhage and infection and the latter, while promising in mice, is limited by the large required dose for a human brain. These methods do not, however, work with the barrier to allow for both delivery and release of molecules to and from the brain microenvironment.

Focused Ultrasound (FUS) is a proposed way to reversibly increase the permeability of the BBB. FUS, when used in conjunction with intravenously-introduced circulating microbubbles, mechanically stimulates blood vessels by causing stable cavitation, or controlled expansion and contraction, of the bubbles when they pass through the low-power ultrasound field. This mechanical stimulus disrupts the tight junctions of endothelial cells and allows for the bidirectional transport of molecules across vessel walls at targeted locations. It is important to note that this is a reversible method and the BBB closes hours after ultrasound exposure thus there is no lasting impact to this therapy (Burgess et al., 2015). Previous research has shown promising results using FUS without neuronal damage (Aryal, Arvanitis, Alexander, & McDannold, 2014), but there is still a need for future experiments that show the effectiveness and extent of exchange caused by FUS-induced BBB disruption and how it varies over time, especially concerning the release of molecules as most studies have focused solely on drug delivery. This is a worthy inquiry because the release of these molecules, such as cell-free circulating tumor DNA (ctDNA), can be used for early diagnosis via blood samples.

Studies indicate that ctDNA collected from the cerebrospinal fluid (CSF) and blood is an informative biomarker that communicates the progression of the tumor (De Mattos-Arruda et al., 2015). There is therefore a need to develop methods that can safely bypass the BBB in order to allow the release of ctDNA into circulation. CtDNA is fragmented DNA found in the noncellular part of blood that is released through apoptosis and necrosis (Corcoran

& Chabner, 2018). It is rapidly cleared from the blood and plasma containing these fragments must therefore be separated from whole blood soon after collection. Mutations in ctDNA can be detected using polymerase chain reaction (PCR) and next-generation sequencing. While some patients may not have enough ctDNA in their blood, which typically happens after starting therapy, the analysis of ctDNA opens new avenues for detection and targeted treatment while mitigating the risks of surgical biopsies. For instance, one study was able to predict which patients of colorectal cancer would eventually relapse (Corcoran & Chabner, 2018). Therefore, if the natural barriers of the CNS can be bypassed to allow detectable levels of ctDNA in the blood, liquid biopsies may be a promising approach to dealing with cancers in these organs.

CHAPTER 2

LITERATURE REVIEW

One of the ways that researchers have been going about GBM is by using animal models for glioma research to further understanding of GBM. Indeed, this is the basis of Oh et al.'s paper in which several murine glioma models are reviewed and compared for immunotherapeutic treatments of human glioblastoma, which would be much less invasive than the current treatments. One of the most widespread and extensively-researched models is the Gl261 murine glioma cell line, which shares many similarities with human glioma, such as mutations in the same genes that are mutated in human cancer. Szatmari et al.'s characterization of Gl261 is one of the most cited sources on this topic and provides the basis for inquiry of the oncogenic gene mutations of the p53 and *ras* genes that are present in this cell line. The mutation on tumor suppressor protein p53 is an especially interesting find as this gene is altered in over half of all human cancer types (Blaszczyk-Thurin, O, & Ertl, 2002) and previous studies have linked the deliberate exposure of UV radiation to create skin cancer in rodents have resulted in mutations in both the p53 gene (Kress et al., 1992), and *–ras* oncogenes (Pierceall, Kripke, & Ananthaswamy, 1992), indicating that mutations of these genes are highly associated with cancer. P53 is a tumor suppressor gene whose function is cell cycle regulation and promotion of apoptosis of cells with abnormal DNA. A mutation in p53 can indicate loss of function and these abnormal cells resist apoptosis and go on to divide and become cancerous. K-ras is an oncogene that, when mutated, does not become deactivated and instead promotes unregulated cell growth, leading to cancer. While the discovery of p53 and k-ras mutations in the Gl261 cell line has been widely accepted and referenced in numerous other studies, it must still be

confirmed in order to use these DNA fragments as viable biomarkers for tumor progression.

CtDNA is secreted by tumors during necrosis and apoptosis and contains tumor-specific genetic information such as these oncogenic fragments that can be used for diagnosis and monitoring of glioblastomas (Paproski, Jovel, Wong, Lewis, & Zemp, 2017). One proposed solution is the analysis of these molecules in circulation that are secreted from the tumors via blood or cerebrospinal fluid samples, a method referred to as a liquid biopsy. Liquid biopsies have the potential to replace traditional tissue biopsies as they can noninvasively communicate vital information about tumors. This DNA contains the genetic information that can be used for detection and diagnosis of tumors. They can also inform about the progression of the tumor such as its reaction or developed resistance to drugs. This information can be used to develop targeted cancer treatments. Since the size of these DNA fragments exceed the maximum size allowed to pass through the BBB, this is another reason why it is important to develop a way to overcome this difficulty. While Burgess' research suggests that FUS is a safe, reversible, and noninvasive way to target specific areas of the brain, experimental data may be lacking to support these claims.

This study aims to evaluate the effectiveness of using FUS to promote the shedding of tumor biomarkers into the circulation using a murine glioma model. In order to monitor the transport of materials into and out of the CNS, Gaussia Luciferase, a highly sensitive bioluminescent enzyme which is easily monitored using available bioanalytical methods, is used. This molecule has the advantage of being quantified using two modalities- bioluminescent imaging, which, while not clinically relevant, allows for a cheap proof-of-concept. The second modality includes genetic amplification and quantification using polymerase chain reaction (PCR). In order to further understanding of human glioma and

explore treatment alternatives, the Gl261 murine glioma cell line will be used to defend or reject the claim that focused-ultrasound-induced blood-brain barrier disruption can increase permeability of this barrier.

CHAPTER 3

METHODS AND MATERIALS

The experimental design of this study included two parallel investigations- one *in vitro* and one *in vivo*. These studies investigate the shedding of two biomarkers into the circulation- Gaussia luciferase and ctDNA. Gaussia luciferase (Gluc) catalyzes the oxidation of its substrate, coelenterazine, in a reaction that produces light. This allows it to be easily monitored using readily-available *in vivo* imaging system (IVIS). In order for murine glioma (Gl261) cells to have this bioluminescent property, a viral-mediated stable transfection was done. First, Gl261 cells were seeded in a 96 - well plate and added concentrations of puromycin antibiotic ranging from 0-5 μg/ml to establish a kill curve and determine the most appropriate selective drug concentration required to kill untransfected cells. Thawed LV-CMV-hGluc-Puro lentivirus from SignaGen was then added to growth media to achieve multiplicity of infection (MOI) 100 for 100,000 cells. One 6- well plate was used with 4 wells with 100,000 cells to be transfected and 2 wells with 100,000 cells control. Polybrene was added to cell culture medium to enhance transfection. Puromycin was added 2 days post-transfection at 4ug/ml concentration and added to fresh medium every other day for one week.

Continuing with the *in vitro* portion of the experiment, DNA from the transfected Gl261-Gluc cells were isolated using the Qiagen DNEasy Blood and Tissue DNA isolation kit to yield isolated DNA at a concentration of 57 ng/ul. This DNA was then used for quantitative polymerase chain reaction (PCR) to amplify the Gluc gene in the DNA using the Stratagene MX3005P thermal cycler, and Universal Probe #123 and FastStart Universal Probe Master (ROX) from Sigma Aldrich. The pure Gluc plasmid was also acquired from SignaGen to be used as a positive control. Two master mixes were created using primer pairs #372

(ACCAGGGGCTGTCTGATCT) and #373 (GGGATGAACTTCTTCATCTTGG) and #368 (TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGACCAGGGGCTGTCTGATC) and #369 (GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGGGATGAACTTCTTCATCTTGG). Each PCR reaction contained 3.8 ul of PCR-grade water, 0.4 ul of forward primer, 0.4 ul of reverse primer, 0.4 ul of probe, 10 ul of master, and 5 ul of DNA. For our negative no-template controls (NTC), PCR-grade water was used in place of DNA. For 7 reactions, serial dilutions of the pure Gluc plasmid was used in ten-fold dilutions ranging from 2 copies to 2 million copies of template per reaction. These were used in place of DNA from the Gl261 cells. There were thus 9 reactions per primer pair, one with NTC, one with our sample DNA, and seven with varying concentrations of Gluc plasmid. Triplicates of each reaction were prepared for a total of 54 reactions. The thermal cycler's initiation phase was set to ten minutes at 95 degrees Celsius, then cycled through denaturation at 95 degrees for 30 seconds, annealing at 55 degrees for 1 minute, and elongation at 72 degrees for 1 minute for 40 cycles.

Another regular PCR experiment was set up in the following way. The same isolated DNA was used in of 1/10, 1/100, 1/1000, 1/10000 dilutions. Positive control dilutions were prepared that contained 1, 10, 100, 1000, and 10000 copies of DNA per reaction. Two negative controls were also prepared in which DNA was used instead of water. A master mix was prepared and distributed equally among 11 PCR tubes each containing 4 microliters of 5X Q5 reaction buffer, 5 microliters forward primer #372, 5 microliters reverse primer #373, 0.4 microliters of dNTP mix, 4 microliters of 5X Q5 High GC Enhancer, 0.2 microliters Q5 Polymerase, 0.4 microliters of water, and 1 microliter of DNA

in the appropriate dilution. The thermal cycler was set to an initial denaturing stage of 98 degrees Celsius for 30 seconds, then cycled through 98 degrees for 10 seconds, 50 degrees for 30 seconds, and 72 degrees for 15 seconds for 33 cycles. After a final extension stage at 72 degrees for 10 minutes, the samples were kept at 4 degrees indefinitely until being loaded in 3% agarose gel electrophoresis and analyzed using a bioanalyzer chip.

For the *in vivo* portion of this study, female albino C57Bl/6 strain mice were injected with transfected Gl261-Gluc cells in the brain via intracranial implantation. Stereotactic implantation was used to inject 3 microliters (500,000 cells) 1 mm posterior and 2 mm to the left of the bregma [10]. After implantation, in vivo imaging system (IVIS) images and blood samples from the tail were collected every 3 days for analysis. The mice were anesthetized in a gas anesthesia chamber (2% isofluorane gas in O_2) until unresponsive. 10 ul of blood was collected from each mouse during each blood sample session, mixed with 2 ul EDTA anticoagulant, and plated on a 96-well plate. This was mixed with a solution containing coelenterazine and imaged using the IVIS machine. After 18 days post-implantation, the mice underwent FUS-induced BBB disruption and all blood was collected for further analysis.

CHAPTER 4

RESULTS

Figure 1 shows the amplification plot for the quantitative PCR delineated in the Methods section. This includes individual plots for each of 54 total reactions. As seen, none of the reactions were showed luminescence above threshold. Therefore, qPCR was unable to amplify the Gaussia Luciferase gene in any of the samples.

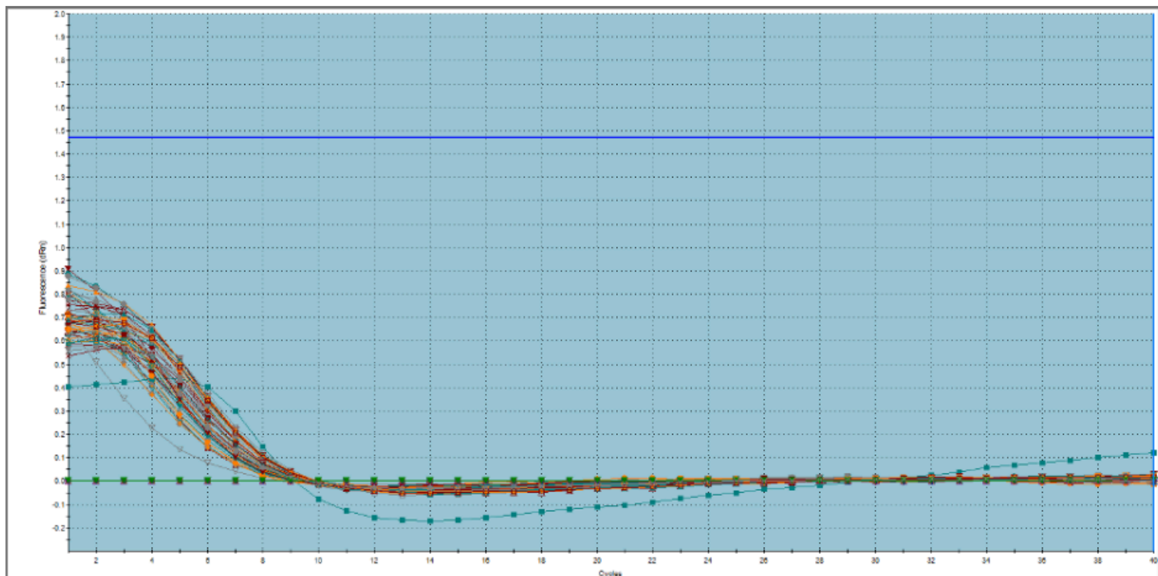

Figure 1: Amplification Plot for Quantitative PCR: *The amplification plots for 54 reactions are shown. The y axis is fluorescence and the x axis is cycles. The blue line is the threshold fluorescence that would give the Ct value for each amplified reaction needed to make a standard curve. This standard curve would allow of the quantification of an unknown. No samples reached this threshold.*

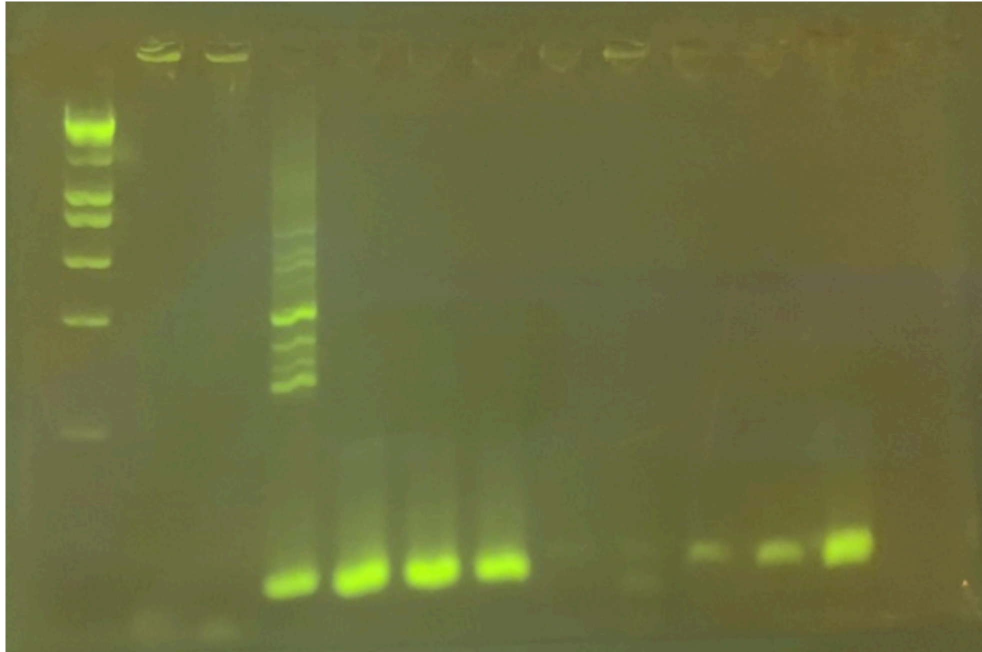

Figure 2: Gel Electrophoresis Result: *The PCR reactions were run on 3% agarose gel. From left to right, the 1st column was loaded with the ladder used to measure the size of the products. The second and third columns were loaded with the no template control reactions. The 4th-7th columns had Gl261 DNA at a 1/10, 1/100, 1/1000, 1/10000 dilutions. The 8th-12th columns had positive control Gluc DNA at 1, 10, 100, 1000, and 10,000 copies of DNA.*

Figure 2 shows the gel electrophoresis result for the regular PCR reaction. The expected PCR products were 100-200 base pairs, which is shown in the gel. From the gel, it can be seen that the reactions worked for both the sample DNA and positive control. The negative control also acted as expected.

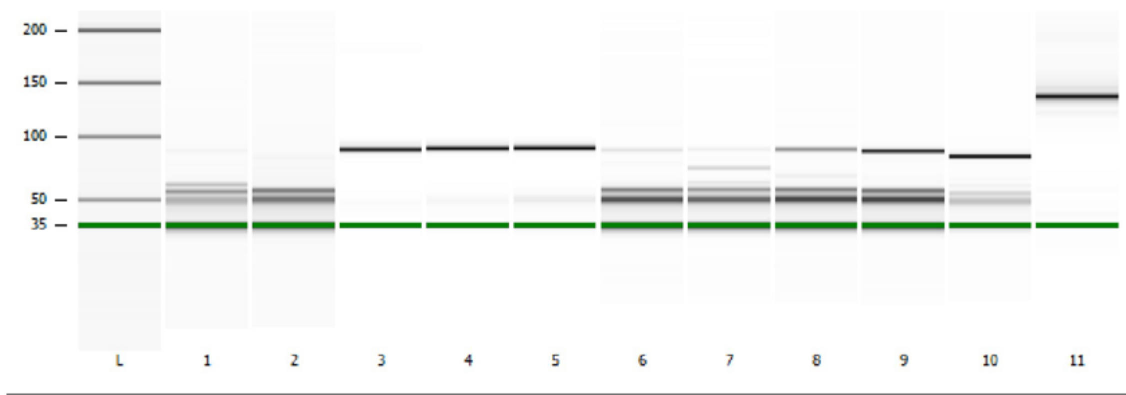

Figure 3: Bioanalyzer Chip Results. The bioanalyzer chip gives more information than the gel electrophoresis. From these results it can be seen that the PCR is highly sensitive as it was able to amplify reactions with a single copy of positive control DNA and the gene was amplified in the 1/10, 1/100, and 1/1000 dilutions of transfected Gl261 DNA.

CHAPTER 5

DISCUSSION AND CONCLUSION

While the quantitative PCR did not amplify the samples as expected, it can be seen that the PCR did indeed work from Figures 2 and 3 and that it is highly sensitive. This is promising as ctDNA is usually found in small quantities and a sensitive method will be needed to detect and quantify it. This also means that the primers work and the DNA was also viable meaning we can use these primers in Illumina sequencing and qPCR, which will require further troubleshooting. The next step after overcoming these difficulties is to compare levels of Gaussia Luciferase before and after Focused Ultrasound treatment, which is the major aim of this study. A future experiment will use blood from the mice who have Gl261-Gluc tumors. The blood will be centrifuged to isolate the plasma and then the DNA from the plasma will be isolated. This will be used for future PCR experiments and quantification of gene expression before and after treatment. While this would be a proof-of-concept that displays that FUS was able to increase the permeability of the BBB and allow ctDNA from the brain microenvironment to enter circulation, Gaussia Luciferase is not a clinically relevant gene. Future studies will target the oncogenic fragments such as mutations on P53 and –ras genes.

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