Cell-free telomere DNA as a biomarker for treatment response and tumor burden in Glioblastoma

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ABSTRACT

Telomeres are repeated nucleotide sequences that cap the ends of each chromosome in eukaryotes. Telomeres are among the earliest genomic sequences to be degraded during apoptosis, potentially providing a biomarker of cell death. To study this, we quantified telomeres in serum from Glioblastoma and non-cancer patients. Cell-free DNA was isolated by centrifugation to remove intact cells, and purified using a QIAamp DNA Blood Midi Kit (Qiagen). Cell-free telomeric (cf-tel) and cell-free actin (cf-actin) DNA were analyzed with quantitative PCR. Total cell-free DNA was measured with PicoGreen assays. We hypothesize that patients with Glioblastoma tumors have higher cf-tel DNA levels than those without tumors. Our results indicate that cf-tel DNA was present at nearly double the amount in Glioblastoma patients when compared to non-cancer control patients with a significant difference ($p=0.0151$), while cf-actin DNA and total cell-free DNA amounts for Glioblastoma patients and non-cancer control patients were nearly identical. We conclude that cell-free telomeric DNA can be detected and measured in serum from normal patients and patients with a history of Glioblastoma, the increased presence of cell-free telomeric DNA is directly correlated with Glioblastoma disease conditions, and cell-free telomeric DNA may be a useful clinical biomarker for treatment response and for measurement of tumor burden.

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LIST OF ABBREVIATIONS

Cf-tel	Cell-Free Telomere
Cf-actin	Cell-Free Actin
GBM	Glioblastoma
qPCR	Quantitative Polymerase Chain Reaction

1. INTRODUCTION

1.1 Glioblastoma

Glioblastoma (GBM) is the most frequent and malignant type of brain tumor. Glioblastomas arise from astrocytes, a gel-like tissue that makes up the supportive tissue of the brain and assumes multiple roles in maintaining an optimally suited environment for neuronal function [1]. The vast majority of Glioblastoma cases, about 90%, are categorized as primary Glioblastoma. Primary Glioblastomas develop de novo, or without clinical or histologic evidence of a less malignant precursor lesion, in older patients with a mean age of 62 years. They are genetically characterized by loss of heterozygosity on chromosome 10q (70% of cases), epidermal growth factor receptor overexpression (36%), p16INK4a tumor suppressor protein deletion (31%), and PTEN gene mutations (25%). Secondary glioblastomas develop through progression from an original, low-grade, non-localized astrocytoma or anaplastic astrocytoma, and manifest in younger patients with a mean age of 45 years [2]. Secondary Glioblastomas are genetically characterized most commonly by TP53 gene mutations, already present in 60% of precursor low-grade astrocytomas. Primary Glioblastomas predominantly affect males, with a population-based male to female ratio of 1.33:1, while secondary glioblastomas primarily occur in females, with a population-based male to female ratio of 0.65:1. Histological criteria for the diagnosis of Glioblastoma include: nuclear atypia, cellular variation of size and shape, mitotic activity, vascular thrombosis, microvascular proliferation, and necrosis [3]. The incidence rate of primary glioblastomas in the United States is 2.96 new cases per 100,000 individuals per year, making it a more rare form of cancer. [2] Glioblastoma symptoms are usually caused by increased pressure in the brain,

necrosis of brain tissue, and seizures caused by the location of the tumor [18]. These symptoms can include headache, nausea, vomiting, and drowsiness. Also, depending on the location of the tumor, patients can develop a variety of other symptoms such as weakness on one side of the body, memory or speech difficulties, and changes in sight [4]. The most frequent location for Glioblastomas are in the cerebral hemispheres, with 95% of these tumors arising in the supratentorial region. Only a small percent of tumors occur in the cerebellum, brainstem, and spinal cord [18]. Glioblastoma is very difficult to treat due to its location and rapid progression. Treatment options include surgery to remove tumor, as well as radiation and chemotherapy to slow the growth of tumors. Prognosis of both primary and secondary Glioblastoma is generally very poor. The majority of patients with primary Glioblastoma (68%) had a clinical history of less than 3 months, with a mean duration of first symptom to diagnosis of 6.3 months. The median overall survival of patients clinically diagnosed with primary Glioblastoma is 4.7 months. The mean duration of the clinical history of patients with secondary Glioblastoma was 16.8 months, with a median survival of 7.8 months, significantly longer due to the younger age of patients diagnosed with secondary Glioblastoma. To date GBM remains incurable due to its heterogeneity and complex pathogenesis. Continued research efforts will help to provide better treatment options to combat the disease in the future [18].

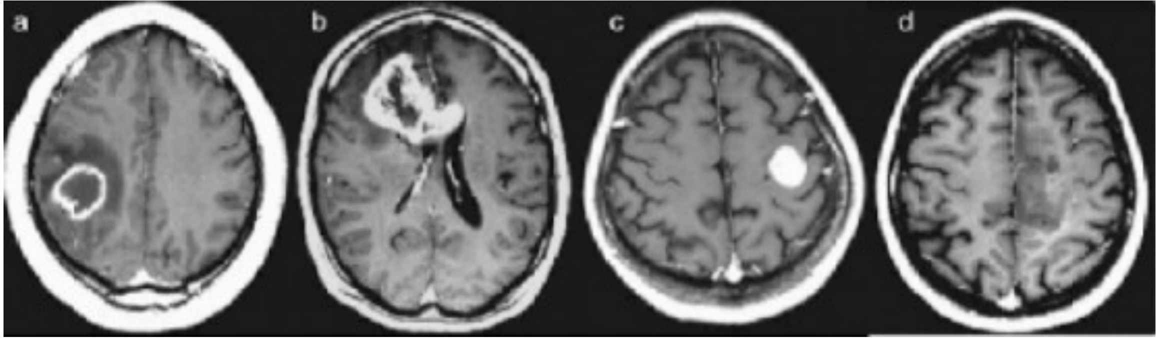

Figure 1: The above image shows four different patients with GBM that illustrate the heterogeneity of the tumor: (a) rim-enhancing mass with central necrosis in the right parietal lobe with surrounding edema; (b) irregularly enhancing mass that crosses the corpus callosum; (c) well-circumscribed homogeneously enhancing mass in the left frontal lobe with no associated edema; (d) ill-defined infiltrative mass in the left medial frontal lobe with no noticeable necrosis [18].

1.2 Telomeres

Telomeres are sequences at the ends of eukaryotic chromosomes that help stabilize the DNA. Telomeres consist of multiple repeated sequences of the form 5'TTAGGG3' in humans, with repeats at the end of strands generally numbering more the 1,500. Telomeres are characterized by a TG leading strand that is longer than its complement, leaving an overhang region of single-stranded DNA of up to a few hundred nucleotides at the 3' end [5]. Telomeres are also coated by a capping protein complex called shelterin. Three shelterin subunits, TRF1, TRF2, and POT1 directly recognize telomeric repeats, and bind with three additional proteins, TIN2, TPP1, and Rap1, to form a complex that allow cells to distinguish telomeres' extended overhang region from sites of double-stranded DNA damage. Without the protective activity of shelterin, telomeres would be inappropriately processed by DNA repair pathways and leave the coding regions of the DNA unprotected [6].

Telomeres protect against threats to the genome that arise from a difficulty inherent in the asymmetric replication of DNA. The ends of linear eukaryotic DNA cannot be routinely replicated by normal cellular replication machinery due to DNA polymerase requiring an RNA primer with a 3′ hydroxyl donor group to initiate DNA replication. Without telomeres, genetic material would be lost every time a cell divides. Therefore, repeated telomeric sequences are added to eukaryotic chromosome ends primarily by the enzyme telomerase [5]. Telomeres and telomerase alleviate this problem by providing a repetitive protective template that can be repaired on the ends of chromosomes, thereby avoiding the loss of genetically encoded information during replication. Telomerase works by containing an RNA component, about 150 nucleotides

long of the form 3’AAUCCC5’, that serves as a RNA template for the synthesis of the repeated 5’TTAGGG3’ DNA strand of the telomere. Telomerase binds to the parental single-stranded overhang and extends the parental strand with a 5’TTAGGG3’ DNA repeat. Telomerase then repositions itself on the parental strand and continuously extends this strand with multiple 5’TTAGGG3’ repeats. After the parental strand has been significantly extended by multiple telomeric repeats, DNA primase adds a RNA primer near the 3’ end and DNA polymerase fills in the vacant region of the telomere. A short overhang on the parental strand will remain; however, the end result is that telomerase will have added many repeat sequences from a few dozen to a few hundred, preventing chromosome ends or coding regions of DNA from shortening.

Efforts to uncover the underlying mechanisms driving genome instability in cancer have revealed a prominent role for telomeres [7]. With subsequent DNA replications in normal cells, telomeric ends continually shorten. As telomere erosion continues, the loss of telomere capping functions produces rampant chromosomal instability and widespread apoptosis. Similarly, shorter telomeres, caused by normal aging, can also induce genomic instability and may contribute to the development of some cancers [8]. Telomerase is used to replicate telomeres and prevent degradation. Yet, despite standard telomerase function, normal cells are still programed to eventually expire. However, in some cancer cells, telomere length is continuously maintained despite multiple cellular divisions due to the overexpression of telomerase reverse transcriptase (TERT), the catalytic protein subunit of telomerase. The ability of telomerase to endow normal cells, which are destined to age and die, with immortal growth potential by preventing the shortening of telomeres has provoked widespread

speculation that telomerase reactivation plays a role in human cancer development. Furthermore, DNA damage-induced apoptosis, which is caused by cell trauma due to conditions like cancer, results in a dramatic telomere loss. It has been shown that cells undergoing apoptosis upon DNA damage also exhibit a rapid and dramatic loss of telomeric sequences [9].

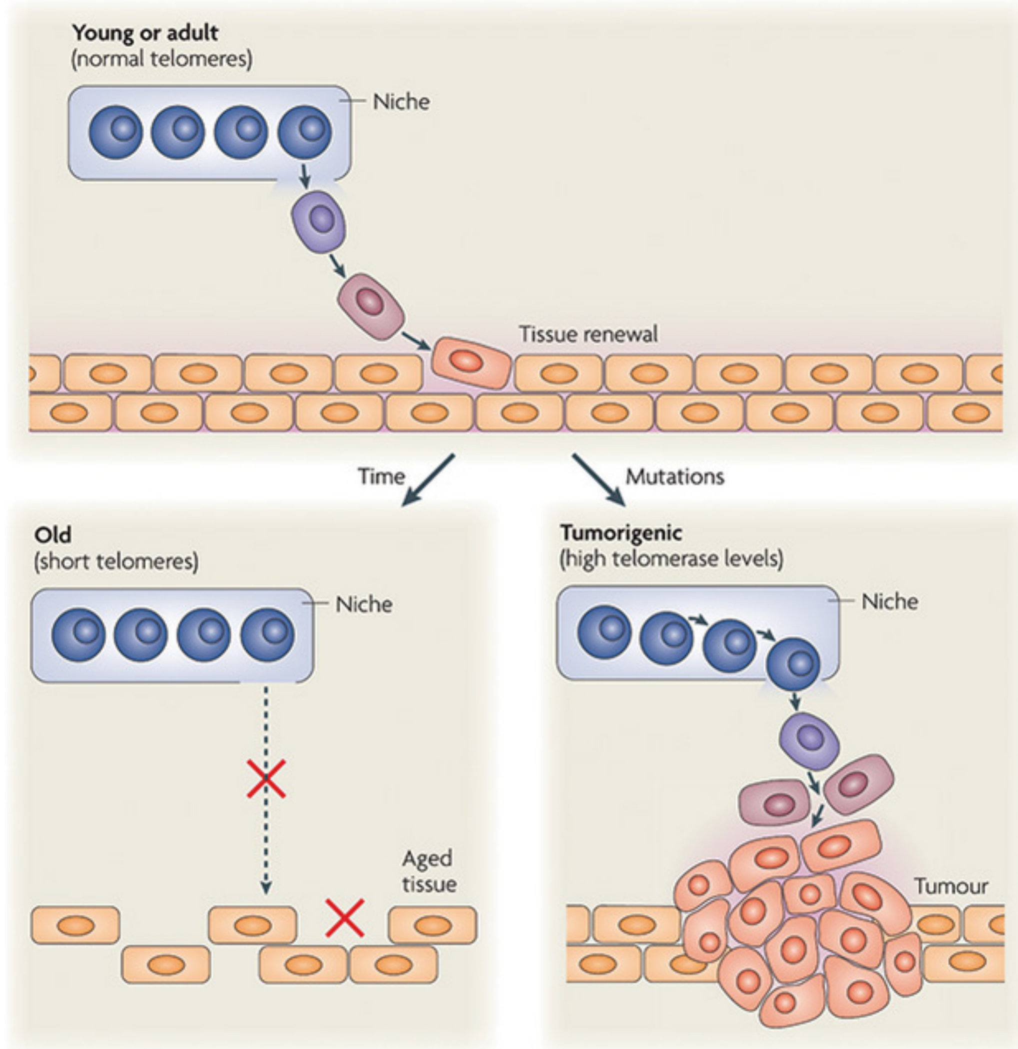

Figure 2: (Top) In normal organisms, groups of stem cells (blue, rounded cells) called niches repair tissues. The stem cells exit the niche, proliferate, and differentiate (square, orange cells). (Bottom left) In older organisms, stem cells have insufficient telomerase activity to maintain telomere integrity. Stem cell telomeres are too short, and consequently tissue regeneration is suboptimal. The ultimate consequence of impaired cell mobilization will be organ failure due to tissue degeneration. (Bottom right) If stem cells express high levels of telomerase, stem cells mobilize more efficiently than normal. Under these conditions, tissues would be maintained longer, therefore increasing life span. However, the probability of forming a tumor is higher [17].

1.3 Cell-Free DNA

Cell-free DNA (or cfDNA) refers to all non-encapsulated DNA in the blood stream [10]. The release of nucleic acids into the blood is related to the apoptosis and necrosis of cells, including that of cancer cells in the tumor microenvironment. Fragments of cellular nucleic acids can also be actively released. It has been estimated that for a patient with a tumor that weighs 100 g, which corresponds to 30 billion tumor cells, up to 3.3% of that tumor's DNA may enter the blood every day. The size of this cell-free DNA varies between small fragments of 70 to 200 base pairs and large fragments of approximately 21 kilobases. Nucleic acids are cleared from the blood by the liver and kidney, and they have a variable life in circulation ranging from 15 minutes to several hours [11].

Tumor cells that circulate in the blood and metastatic deposits that are present at distant sites can further contribute to the release of cfDNA. Increased levels of circulating nucleic acids (DNA, mRNA and microRNA (miRNA)) in the blood reflect pathological processes, including malignant and benign lesions, inflammatory diseases, stroke, trauma and sepsis. In cancer patients, circulating DNA carries tumor-related genetic and epigenetic alterations that are relevant to cancer development, progression and resistance to therapy. The cellular source of tumor-derived circulating nucleic acids is still subject to debate. After complete removal of the primary tumor, the detection of cfDNA may signal the presence of micrometastatic cells in distant organs, which pose a risk of relapse. Minimally invasive blood analyses of cell-free DNA allow repetitive, real-time monitoring of metastatic changes and will, therefore, gain clinical utility in the determination of prognosis and treatment efficacy. Cell-free DNA in plasma or serum has

the clinical potential to become a more specific tumor marker for the diagnosis and prognosis, as well as the early detection, of cancer [11]. Measuring cell-free DNA may complement currently used tumor markers for the management of cancer patients. Cell-free DNA has been described as a viable biomarker of several cancers across multiple studies [12,13,14,15]. Circulating tumor DNA is an informative, inherently specific, and highly sensitive biomarker of metastatic cancer [20].

2. METHODS

2.1 Patient Samples

This research was conducted at the headquarters of Blondin Bioscience in Birmingham, AL under the direct supervision of Dr. Katri Selander, Dr. Kevin Harris, Mr. Brad Spencer, and Mr. Kevin Stoltz. Fifty patient samples, provided by the University of Alabama-Birmingham, were used for this study. Forty samples contained the serum collected from blood samples of various patients across different stages of primary Glioblastoma. Nine samples contained the serum collected from blood samples of various non-cancer patients with Epilepsy. Samples were collected between 2008 and 2015, and were immediately frozen until used to prevent DNA degradation. The identity, clinical information, and outcome of these patients were unknown due to HIPAA laws.

2.2 DNA Isolation

The 1000-μL serum samples were centrifuged at 13000 rpm for 5 minutes in a microcentrifuge at room temperature to remove cells and large cellular debris. 500 μL of the supernatant liquid was carefully removed from each sample, and the supernatant liquid was centrifuged again in a microcentrifuge at 13,000 rpm for 5 minutes at room temperature to further remove cellular debris. 400 μL of the supernatant liquid from the second centrifugation was removed and frozen until time for DNA isolation. DNA isolation of the cell-free samples was done with a QIAamp DNA Blood Midi Kit (Qiagen) using the following standard protocol. 40 μL Qiagen Protease was added to a clean 1.5 mL microcentrifuge tube. 400 μL of serum sample was added to the tube. 400 μL of AL buffer was added to the tube, and the tube was pulse vortexed for 15 seconds to

lyse the cells. After pulse vortex, the mixture was incubated for 10 minutes at 56 °C to achieve the maximum DNA yield. Next, 400 µL of ethanol (96-100%) was added, and the tube was pulse vortexed again for 15 seconds. 500 µL of the subsequent mixture was added to a Midi Spin Column, and spun in a microcentrifuge at 13000 rpm for 1 minute. The contents collected in the collection tube were discarded. Afterwards, the Midi Spin Column was re-spun at 13000 rpm for 1 minute, and collection tube contents were discarded again to further remove any unwanted contaminates. Next, 750 µL of wash buffer AW1 was added to the Midi Spin Column, and spun in a microcentrifuge at 13000 rpm for 1 minute. The contents collected in the collection tube were discarded. Afterwards, the Midi Spin Column was re-spun at 13000 rpm for 1 minute, and collection tube contents were discarded again to further remove any unwanted contaminates. This process was repeated exactly for wash buffer AW2. Afterwards, the Midi Spin Column was spun once again at 13000 rpm for 1 minute to dry the membrane, and the collection tube was discarded. Lastly, the Midi Spin Column was placed on a clean 1.5 mL microcentrifuge tube. 400 µL of elution buffer AE was added to the column, and allowed to incubate for 1 minute at room temperature. The column was then spun at 8000 rpm for 1 minute and the isolated DNA was collected in the 1.5 mL tube.

2.3 Quantitative PCR

Cell-free telomeric DNA was measured using Cawthon Quantitative PCR [16]. A nine step, 4x serial dilution DNA standard curve ranging from 1500-0.02 ng/mL created using human genomic DNA was used for this assay. A specific combination, or cocktail, of DNA, either standard curve or experimental, nuclease-free DI water, forward and

reverse qPCR primers, and 2x All-in-One qPCR mastermix was plated in triplicate for each unknown DNA sample in a 96-well plate. Thermal cycling conditions for cf-tel DNA were 2 min at 50 °C, 10 min at 95 °C, 26 cycles of 15 sec at 96 °C and 2 min at 54 °C, 2 min at 95 °C, 2 min at 50 °C, 15 sec at 95 °C, 15 sec at 60 °C, and 15 sec at 95 °C. Thermal cycling conditions for cf-actin DNA were 2 min at 50 °C, 10 min at 95 °C, 40 cycles of 15 sec at 96 °C and 1 min at 60 °C, 2 min at 95 °C, 2 min at 50 °C, 15 sec at 95 °C, 15 sec at 60 °C, and 15 sec at 95 °C. The use of normal genomic DNA for a standard curve allowed us to directly determine relative cf-tel and cf-actin DNA concentrations in the test samples.

2.4 PicoGreen Analysis

Total cell-free DNA was measured using a Quant-iT PicoGreen fluorescent assay. A 4-step, 10x serial dilution DNA standard curve ranging from 1000-1 ng/mL created using lambda DNA was used for this assay. A specific combination of DNA, either standard curve or experimental, and PicoGreen reagent, composed of Quant-iT PicoGreen dsDNA reagent and TE buffer, was plated into a 96-well microplate. A Cary eclipse fluorescence spectrophotometer measured the absorbance of each DNA sample. Samples were excited at a wavelength of 480 nm and emissions were measured at a wavelength of 520 nm. Once all absorbance values were measured, concentrations were determined based off of a graph of the standard curve's absorbance vs. concentration shown in Figure 3.

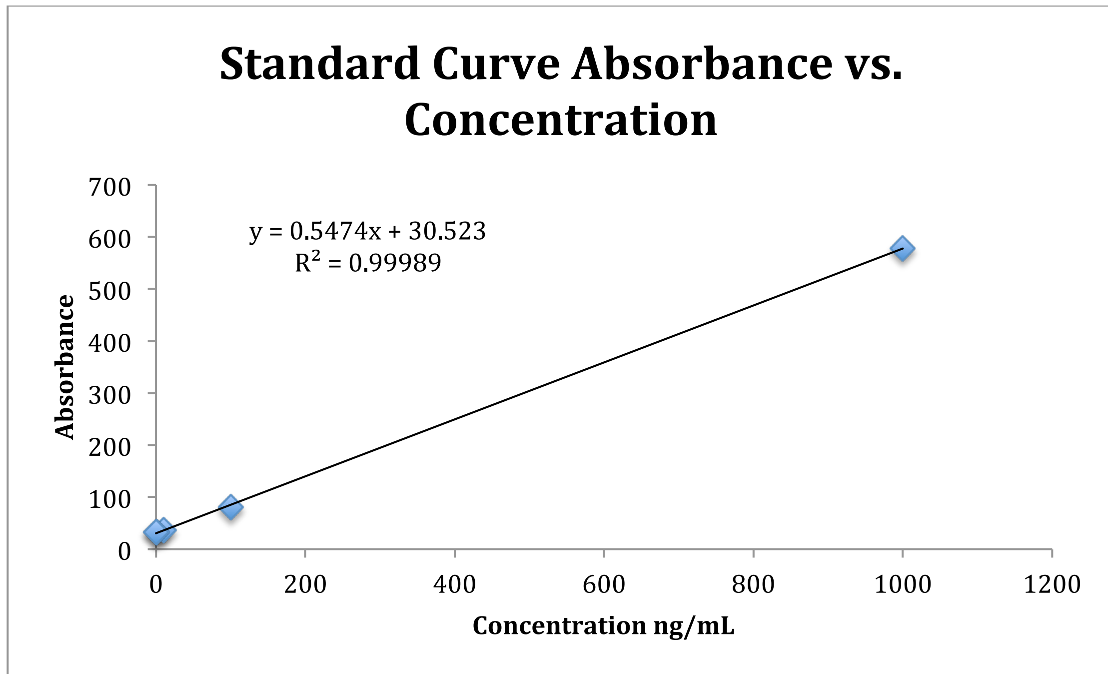

Figure 3: Graph of Absorbance vs. Concentration based on the standard curve created to determine the total DNA concentrations of each unknown sample. Our y-intercept for this graph ideally should be 0, as our blank should not have fluoresced. However, our absorbance reading for our blank measured 32.332, explaining the cause of our raised y-intercept. Another issue concerning the accuracy of our results is that the majority of our calculated total cell-free DNA concentrations fall within the lower range of our standard curve. One way to improve the accuracy of this test would be to adjust the standard curve by only measuring the absorbance of multiple lower concentrations of lambda DNA to more accurately analyze lower end total cell-free DNA concentrations.

2.5 Statistical Procedure

PRISM 7 (GraphPad) was used to conduct all statistical analyses. Standard error of measurement was determined. Unpaired t-tests were used to determine statistical significance at a level of $P<0.05$. A p-value of 0.05 signifies that there is only a 5% chance that the values analyzed are different due to mere random chance, strongly suggesting that there is some other mechanism responsible for the difference between the two groups. Due to a wide range in our results, some data points were excluded from statistical analysis as outliers. The criteria for selecting these outliers was if the value of the data point was at least 10-fold greater than other values of the same category the data point was considered an outlier. For this set of experiments we found and excluded four outliers.

3. RESULTS

3.1 Summary of Results

Our experiments focused on successfully quantifying the amount of cell-free telomeric DNA in cancer patients. We hypothesized that a) cell-free telomeric DNA is released in significantly higher amounts in patients with Glioblastoma than non-cancer control patients, b) other cell-free DNA measurements would not have significant differences between cancer and non-cancer patients, c) cell-free telomeric DNA would be preferentially released in cancer patients compared to other cell-free DNA microparticles (cf-actin). Table 1 summarizes the result from this research.

3.2 Cell-free telomere DNA

To determine if cell-free telomeric DNA is released in significantly higher amounts in Glioblastoma patients, we used qPCR to quantify the amount of cf-tel DNA released by 40 Glioblastoma patients and compared it to the amount of cf-tel DNA released by 9 non-cancer patients. Each sample was run in triplicate to ensure accurate readings and to decrease the possibility of human error. Detectable amounts of cf-tel DNA were measured in both sets of patients. An outlier in sample 3 of the control group, which measured a cf-tel DNA mean amount over 30-fold greater than the other samples, was noticed and removed before statistical analysis. After collecting data, we determined the mean level of cf-tel DNA in Glioblastoma patents to be 2.063-ng/mL ± 0.1526, and the mean level of cf-tel in non-cancer patients to be 1.185-ng/mL ± 0.1167, a nearly 2-fold increase in difference between Glioblastoma and non-cancer patients. We subsequently ran a two-tailed, unpaired t-test on the mean levels of cf-tel DNA, and

Table 1: Summary of the results of qPCR and PicoGreen Analysis. All concentrations are measured by ng/mL. Each sample is representative of a single patient either in the control or Glioblastoma group. Concentrations of cf-tel and cf-actin DNA are averages of measurements made in triplicate.

Sample	Average cf-tel DNA	Average cf-actin DNA	Total cell-free DNA
C1	1.44	2.43	43.04
C2	1.24	1.98	41.32
C3	30.87	14.29	557.33
C4	1.25	1.44	35.44
C5	1.08	2.12	50.9
C6	1.81	1.26	50.97
C7	0.98	1.80	43.55
C8	0.82	1.38	68.98
C9	0.85	1.54	100.09
GBM1	1.73	1.78	44.15
GBM2	1.04	1.62	51.32
GBM3	2.22	2.35	49.84
GBM4	1.40	1.64	46.2
GBM5	0.57	1.68	49.45
GBM6	5.27	16.73	134.02
GBM7	1.32	1.87	45.51
GBM8	1.77	2.36	80.38
GBM9	2.01	1.44	69.82
GBM10	2.81	2.58	58.64
GBM11	1.63	2.35	49.51
GBM12	3.38	2.39	76.11
GBM13	0.60	1.11	43.06
GBM14	1.17	0.84	52.96
GBM15	3.02	3.06	68.29
GBM16	2.99	1.32	44.56
GBM17	3.41	2.30	52.16
GBM18	2.22	1.11	59.12
GBM19	3.02	0.89	49.47
GBM20	2.43	1.07	43.41
GBM21	2.01	1.49	56.56
GBM22	1.72	1.98	45.73
GBM23	1.63	0.80	66.19
GBM24	1.81	1.09	56.05
GBM25	1.75	0.64	40.3
GBM26	3.27	5.36	59.68
GBM27	2.75	4.19	65.35
GBM28	1.75	0.79	51.79
GBM29	1.31	0.82	55.37
GBM30	1.08	1.04	53.65
GBM31	1.20	0.93	67.63
GBM32	2.57	0.55	65.07
GBM33	1.82	1.60	59.92
GBM34	1.38	1.61	52.59
GBM35	2.15	0.58	61.2
GBM36	2.19	0.78	51.32
GBM37	2.95	4.20	57.07
GBM38	0.83	1.05	44.94
GBM39	3.49	0.71	36.54
GBM40	0.81	1.13	40.08

achieved significant results (p=0.0151), signifying that the difference between cf-tel DNA levels between cancer and non-cancer patients was not due to chance. This supports our hypothesis that cell-free telomeric DNA is released in significantly higher amounts in patients with Glioblastoma than non-cancer control patients, making it a viable biomarker for assessment of Glioblastoma patients. The measurements of cf-tel DNA for each sample and differences between Glioblastoma and non-cancer patient's levels are shown below in Figure 4.

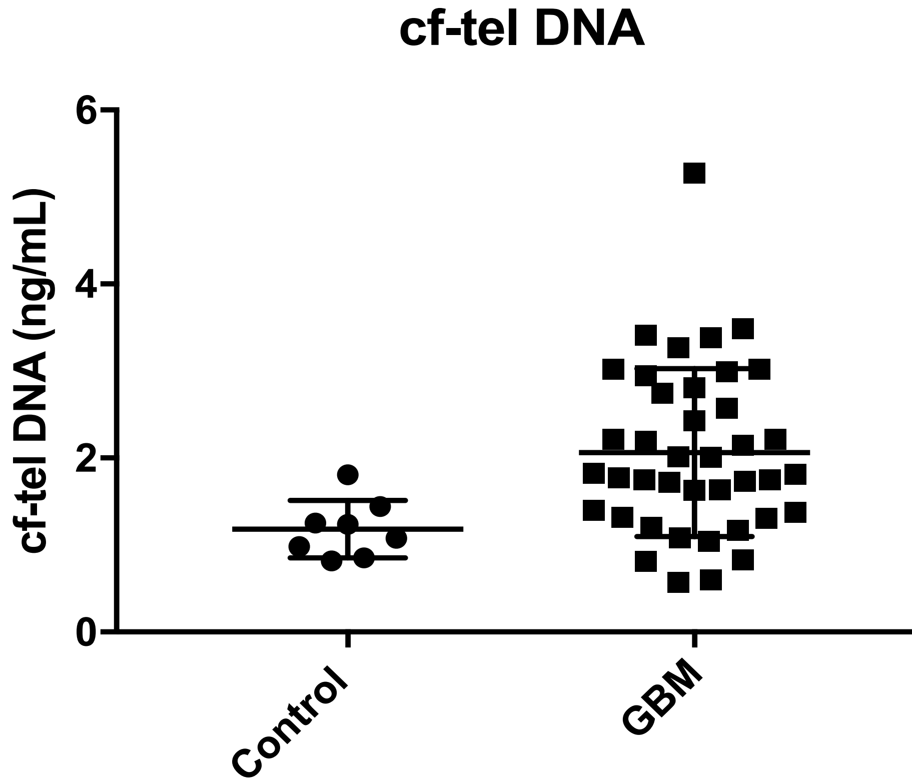

Figure 4: Cell-free telomeric DNA is detectable in both Glioblastoma and non-cancer patents. Results are expressed as total relative telomere amounts as calculated from a normal human genomic DNA standard curve. Bars indicate the mean value of each group, 2.063-ng/mL for GBM and 1.185-ng/mL for Control. There was a significant difference between the mean value for the GBM group and the control group (p=0.0151).

3.3 Cell-free actin and total cell-free DNA

Next, to determine if other cell-free DNA measurements were significantly different between cancer and non-cancer patients, we used qPCR and PicoGreen analysis to quantify the amount of cell-free actin DNA and total cell-free DNA, respectively, in the same 40 Glioblastoma and 9 non-cancer control patients. For the cf-actin qPCR, each sample was run in triplicate to ensure accurate readings and to decrease the possibility of human error. For total cell-free DNA PicoGreen analysis, each sample was run once. Detectable amounts of cf-actin and total DNA were measured in both sets of patients. Three outliers were noticed and removed before statistical analysis. Two of the outliers were the measurements for control sample 3 of both cf-actin and total cell-free DNA, which measured a cf-actin DNA mean amount about 13-fold greater and a total cell-free DNA mean amount about 10-fold greater than the other respective samples. The other outlier was the measurement for Glioblastoma sample 6 of cf-actin, which measured a cf-actin DNA mean amount about 15-fold greater than the other respective samples. After collecting data, we determined the mean level of cf-actin DNA in Glioblastoma patents to be 1.67-ng/mL ± 0.1713, and the mean level of cf-actin in non-cancer patients to be 1.743-ng/mL ± 0.144. We determined the mean level of total cell-free DNA in Glioblastoma patents to be 56.38-ng/mL ± 2.542, and the mean level of total cell-free DNA in non-cancer patients to be 54.29-ng/mL ± 7.442. We subsequently ran a two-tailed, unpaired t-test on the mean levels of cf-actin DNA and total cell-free DNA, and did not achieve significant results for either ($p=0.8504$ for cf-actin DNA and $p=0.7515$ for total cell-free DNA), signifying that the difference between both cf-actin DNA levels and total cell-free DNA levels between Glioblastoma and non-cancer patients was due to

chance. This supports our hypothesis that other cell-free DNA measurements, cf-actin and total cell-free DNA, would not have significant differences between cancer and non-cancer patients, making cf-tel DNA a unique biomarker for the measurement of tumor burden and treatment response. The measurements of cf-actin DNA for each sample, and differences between Glioblastoma and non-cancer patient's levels, are shown below in Figure 5. The measurements of total cell-free DNA for each sample, and differences between Glioblastoma and non-cancer patient's levels, are shown below in Figure 6.

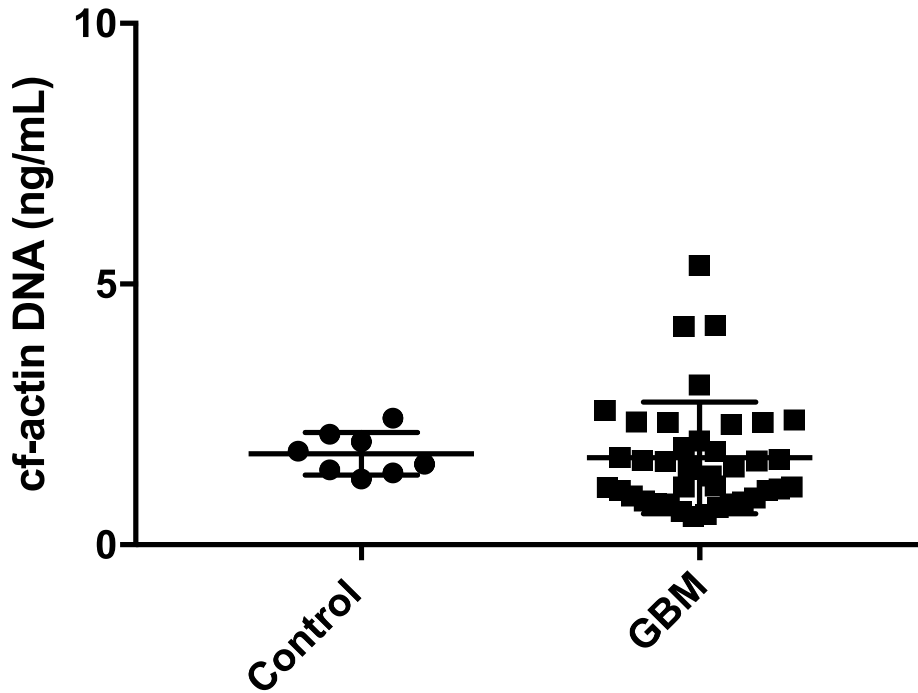

Figure 5: Cell-free actin DNA is detectable in both Glioblastoma and non-cancer patents. Results are expressed as total relative telomere amounts as calculated from a normal human genomic DNA standard curve. Bars indicate the mean value of each group, 1.67-ng/mL for GBM and 1.743-ng/mL for Control. There was no significant difference between the mean value for the GBM group and the control group (p=0.8504)

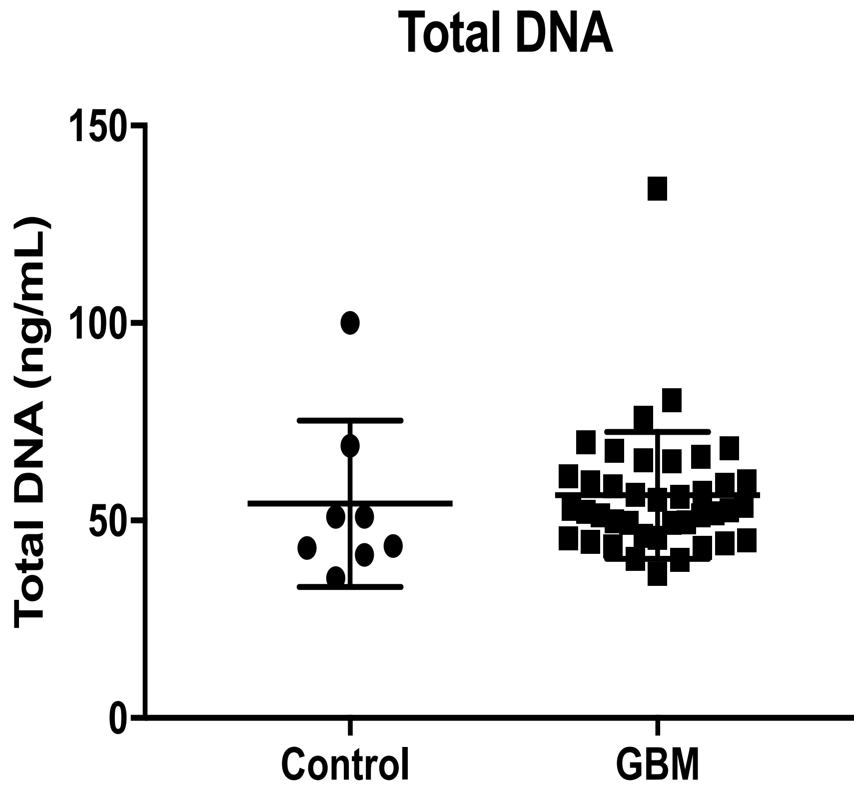

Figure 6: Total cell-free DNA is detectable in both Glioblastoma and non-cancer patents. Results are expressed as total relative telomere amounts as calculated from a lambda DNA standard curve. Bars indicate the mean value of each group, 56.38-ng/mL for GBM and 54.29-ng/mL for Control. There was no significant difference between the mean value for the GBM group and the control group (p=0.7515)

3.4 Comparison of cf-tel and cf-actin

Lastly, to determine if cell-free telomeric DNA would be preferentially released in the Glioblastoma microenvironment compared to other cell-free DNA microparticles (cf-actin), we compared the results of cf-tel and cf-actin qPCR to determine which microparticle was released in higher amounts for Glioblastoma patients. An outlier for cf-actin in Glioblastoma sample 6, which measured a cf-actin DNA mean amount about 15-fold greater than the other samples, was noticed and removed before statistical analysis. After analyzing data, we determined the mean level of cf-tel DNA in Glioblastoma patents to be 2.063-ng/mL ± 0.1526, and the mean level of cf-actin in Glioblastoma patients to be 1.67-ng/mL ± 0.1713. We subsequently ran a two-tailed, unpaired t-test on the mean levels of cf-tel DNA vs. cf-actin DNA in Glioblastoma patients, and unfortunately did not achieve significant results ($p=0.0903$), signifying that the difference between cf-tel DNA levels and cf-actin DNA levels in Glioblastoma patients was due to chance under our set of parameters. However, if the alpha level of our test is changed from $\alpha=0.05$ to $\alpha=0.1$, the test is changed to a one-tailed t-test ($p=0.0452$), or if a few data points are removed from analysis, results become significant. Also, the prevalence of cf-tel DNA over cf-actin DNA in Glioblastoma patients is further suggested when these results are compared to the results for the non-cancer control group, where cf-actin DNA is present in significantly higher amounts than cf-tel DNA ($p=0.0093$), signifying that the difference between cf-tel DNA levels and cf-actin DNA levels in non-cancer patients are not due to chance. However, these results should not be trusted due to the small sample size ($n=8$). Overall, this indicates that if additional subjects were added to the study, a more definitive conclusion could be drawn. Had significant results been found, they

would have supported our hypothesis that cell-free telomeric DNA would be preferentially released in cancer patients compared to other cell-free DNA microparticles. The measurements of cf-tel DNA and cf-actin DNA for each sample and differences between cf-tel DNA and cf-actin DNA in Glioblastoma patient's levels are shown below in Figure 7. The measurements of cf-tel DNA and cf-actin DNA for each sample and differences between cf-tel DNA and cf-actin DNA in non-cancer control patient's levels are shown below in Figure 8.

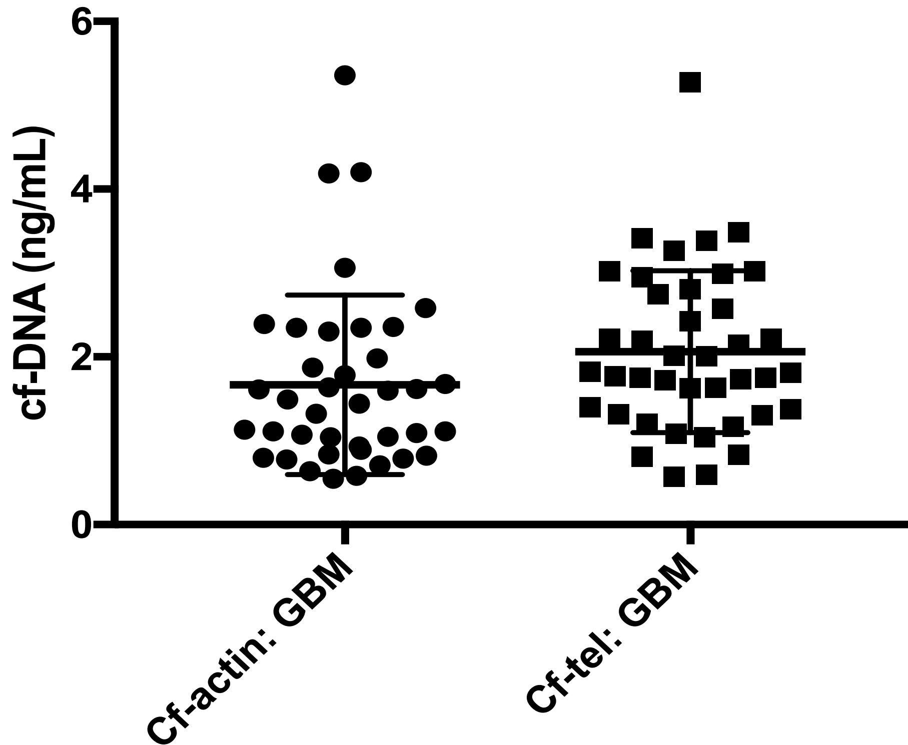

Figure 7: Cell-free telomeric and cell-free actin DNA is detectable in Glioblastoma patents. Results are expressed as total relative telomere amounts as calculated from a normal human genomic DNA standard curve. Bars indicate the mean value of each group, 2.063-ng/mL for cf-tel and 1.67-ng/mL for cf-actin. There was no significant difference between the mean value for the cf-tel GBM group and the cf-actin GBM group (p=0.0.0903)

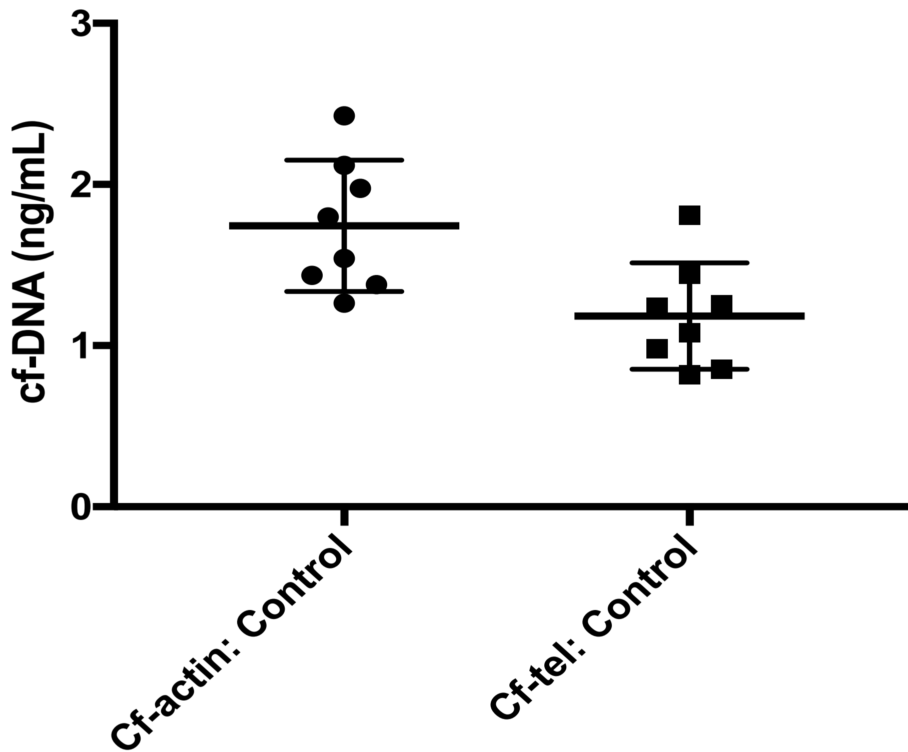

Figure 8: Cell-free telomeric and cell-free actin DNA is detectable in non-cancer patents. Results are expressed as total relative telomere amounts as calculated from a normal human genomic DNA standard curve. Bars indicate the mean value of each group, 1.185-ng/mL for cf-tel and 1.743-ng/mL for cf-actin. There was a significant difference between the mean value for the cf-tel Control group and the cf-actin Control group (p=0.0.0093)

4. DISCUSSION

Our project aimed to be a preliminary study to determine the likelihood and show evidence of measurable cf-tel DNA in Glioblastoma patient blood serum. We hypothesized that a) cell-free telomeric DNA is released in significantly higher amounts in patients with Glioblastoma than non-cancer control patients, b) other cell-free DNA measurements would not have significant differences between cancer and non-cancer patients, and c) cell-free telomeric DNA would be preferentially released in cancer patients compared to other cell-free DNA microparticles (cf-actin). These hypotheses point towards the idea that cf-tel DNA could be an effective and unique biomarker for treatment response and tumor burden in Glioblastoma patients. We predict that plasma cf-tel DNA levels will decrease with time during effective cancer treatment, but will not decrease with time during ineffective cancer treatment. We predict that plasma cf-tel DNA levels also surge upon tumor evolution or metastasis, treatment-resistant clones emerging, and in the case of cancer relapse. Lastly, within the clinical setting, measuring cf-tel DNA would enable oncologists to rapidly assess chemotherapy regimens for patients, getting them to the optimum treatment pathway sooner and in a more cost-effective manner than traditional tumor size imaging.

In our project, we show that telomeric DNA can be detected and quantified in the serum of cancer patients and healthy individuals. We also discovered that cf-tel DNA was present in significantly greater amounts in the serum of Glioblastoma patients compared to that of non-cancer patients, suggesting that cf-tel DNA is released in measurably higher amounts in Glioblastoma patients than non-cancer patients due to the disease conditions. This finding was not similar for cf-actin or total cell-free DNA. Due to the

inability to replicate the findings of cf-tel DNA in cf-actin or total cell-free DNA, we can conclude that cf-tel DNA could be a unique biomarker for the measurement of tumor burden and treatment response in Glioblastoma patients, as other cell-free DNA measurements were unable to detect significant difference between Glioblastoma and non-cancer environments.

One possible mechanism for the preferential release of cf-tel DNA is increased apoptosis-mediated DNA degradation of the telomere region in particular compared to other genomic regions. As stated in the introduction, DNA damage-induced apoptosis caused by cell trauma due to conditions like cancer results in a dramatic telomere loss [9]. Furthermore, the apoptosis and necrosis of cells are related to the release of nucleic acids into the blood [11]. Therefore, Glioblastoma mediated apoptosis and necrosis of cells would lead to the preferential release of telomeric DNA into the blood, causing the measurably elevated levels of cf-tel DNA in Glioblastoma patients.

There was a seemingly large range of values in both Glioblastoma and non-cancer patients. We suspect that this indicates variations in normal activities during daily life, and potentially changes in the pathology of the disease. Nucleic acids are cleared from the blood by the liver and kidney, and they have a variable half-life in the circulation ranging from 15 minutes to several hours [11]. Therefore, even in healthy, non-cancer individuals it is possible to have spikes in cf-tel DNA release caused by DNA damage due to normal activities such as minor injuries or acute illness, making the timing of the collection of samples very important. This proved true for our data, as the ranges in values of cf-tel DNA in non-cancer patients included a few outliers that could have been caused by acute cellular trauma. However, if this is the case, changes in pathology such

as tumor evolution, treatment-resistant clones emerging, and cancer relapse could increase the amount of cf-tel DNA in plasma. This variation also proved true for our study, as the range in cf-tel measurements for the Glioblastoma patients could have been caused by patients at different stages of tumor development and treatment. A potential future study could compare the values of cf-tel DNA to patient staging information and determine if higher levels of cf-tel correlated to more aggressive and developed Glioblastoma tumors as we have predicted. This was not possible in our study as patient information was withheld from researchers. Other potential future studies could include cell culture and animal model experiments to visualize the effects of different chemotherapy regiments on the release of cf-tel DNA.

Practicing oncologists and professional clinical societies are currently emphasizing the need for improved biomarkers for cancer treatment monitoring. Tumor biopsies have been and currently are the keystone for biomarker testing. However, some limitations of tumor biopsies are still significant, such as difficulties in obtaining tissue samples, and the invasiveness of the process [19]. Glioblastoma is an extremely rapidly progressing form of cancer that is located in a difficult region to biopsy. Glioblastoma is treated by surgery to remove tumor, as well as radiation and chemotherapy to slow the growth of tumors. Because the disease progresses rapidly, accuracy and efficiency in the development of a treatment plan are crucial to the outcome of the patient. Taken together, this suggests that there is a need for rapid, inexpensive, cell-free blood biomarker that can help quickly determine if a specific chemotherapy regimen is working, and to detect the presence of metastatic deposits that are present at distant sites. This would enable the clinician to limit exposure to ineffective regimens and decrease side effects, cost, and

wasted time for the patient. It also would confirm the complete removal of tumors, and alert physicians to further actions that may need to be taken elsewhere in the body. The measurement of serum cf-tel DNA during chemotherapy treatment and after surgery may aid this decision-making process. Further studies of cf-tel DNA could lead to the development of a unique cancer biomarker for cell death in vivo. A rise in cf-tel DNA could be an omen for early relapse, thus allowing this to be a sensitive minimal residual disease marker for diagnosis in the future.

5. CONCLUSIONS

This thesis project had the goal of quantifying the concentrations of telomeres in serum from Glioblastoma and non-cancer patients. Our results indicated that cf-tel DNA was present at nearly double the amount in Glioblastoma patients when compared to non-cancer control patients with a significant difference (p=0.0151), while cf-actin DNA and total cell-free DNA amounts for Glioblastoma patients and non-cancer control patients were nearly identical. This suggests that cell-free telomeric DNA can be detected and measured in serum from normal patients and patients with a history of Glioblastoma, and that the increased presence of cell-free telomeric DNA can be directly correlated with Glioblastoma disease conditions. We can infer from these results that cell-free telomeric DNA may be useful as a clinical biomarker for treatment response and for measurement of tumor burden. This research should result in a publication within the next few years.

6. REFERENCES

1. Sidoryk-Wegrzynowicz, M., Wegrzynowicz, M., Lee, E., Bowman, A. B., & Aschner, M. (2011). Role of Astrocytes in Brain Function. *Toxicologic Pathology*, 39, 115-123.

2. Ohgaki, H., & Kleihues, P. (2007). Genetic Pathways to Primary and Secondary Glioblastoma. *The American Journal of Pathology*, 170(5), 1445-1453. Retrieved from PMC.

3. Ohgaki, H., & Kleihues, P. (2012). The Definition of Primary and Secondary Glioblastoma. *Clinical Cancer Research*.

4. Glioblastoma (GBM). (2012). In *American Brain Tumor Association*. Retrieved from www.abta.org/brain-tumor-information/types-of-tumors/glioblastoma.html

5. Nelson, D. L., & Cox, M. M. (2013). *Lehninger Principles of Biochemistry* (6th ed., pp. 984-985, 1026). New York, NY: W. H. Freeman and Company.

6. de Lange, T. (2005). Shelterin: the protein complex that shapes and safeguards human telomeres. *Genes and Development*, 19, 2100-2110. Retrieved from NCBI.

7. Artandi, S. E., & DePinho, R. A. (2010). Telomeres and telomerase in cancer. Carcinogenesis, 31(1), 9-18. doi:10.1093/carcin/bgp268

8. Shammas, M. A. (2011, January). Telomeres, lifestyle, cancer, and aging. *Cure Opin Clin Nutr Metab Care*, 14(1), 28-34. doi:10.1097/MCO.0b013e32834121b1

9. Ramírez, R., Carracedo, J., Jiménez, R., Canela, A., Herrera, E., Aljama, P., & Blasco, M. A. (2003, January 10). Massive Telomere Loss Is an Early Event of DNA Damage-induced Apoptosis. *The Journal of Biological Chemistry*, 278(2), 836-842. doi:10.1074/jbc.M206818200

10. Brune, G. (2017, February). What is Cell Free DNA (cfDNA)? . In *ThermoFischer Scientific*. Retrieved from https://www.thermofisher.com/blog/behindthebench/what-is-cell-free-dna-cfdna-seq-it-out-19/

11. Wu, T., Zhang, D., Chia, J., Tsao, K., Sun, C., & Wu, J. T. (2002, July). Cell-free DNA: measurement in various carcinomas and establishment of normal reference range. *Clinica Chimica Acta*, 321(1-2), 77-87. Retrieved from NCBI.

12. Jung, K., Fleischhacker, M., & Rabien, A. (2010, November 11). Cell-free DNA in the blood as a tumor biomarker-A critical appraisal of the literature. *Clinica Chimica Acta*, 411(21), 1611-1624. Retrieved from Science Direct.

13. Ellinger, J., Müller, S. C., Stadler, T. C., Jung, A., von Ruecker, A., & Bastian, P. J. (2011, April). The role of cell-free circulating DNA in the diagnosis and prognosis of prostate cancer. *Urologic Oncology: Seminars and Original Investigations*, 29(2), 124-129. Retrieved from Science Direct.

14. Canzoniero, J. V., & Park, B. H. (2016, April). Use of cell free DNA in breast oncology. *Biochimica et Biophysica Acta (BBA) - Reviews on Cancer*, 1865(2), 266-274. Retrieved from Science Direct.

15. Idei, T., Sakamoto, H., & Yamamoto, T. (2002, June 10). Terminal restriction fragments of telomere are detectable in plasma and their length correlates with clinical status of ovarian patients. *Journal of internal Medical Research*, 278(2), 836-842. doi:10.1177/147323000203000304

16. Cawthon, R. M. (2009). Telomere length measurement by a novel monochrome multiplex quantitative PCR method. *Nucleic Acids Research*, 37(3), 21e. doi: 10.1093/nar/gkn1027

17. Serrano, M. & Blasco, M. A. (2007). Cancer and aging: convergent and divergent mechanisms. *Nature Reviews Molecular Cell Biology 8*, 715–722. doi: 10.1038/nrm2242

18. Hanif, F., Muzaffar, K., Perveen, K., Malhi, S. M., & Simjee, S. U. (2017). Glioblastoma Multiforme: A Review of its Epidemiology and Pathogenesis through Clinical Presentation and Treatment. *Asian Pacific Journal of Cancer Prevention*, 18(1), 3-9. doi:10.22034/APJCP.2017.18.1.3

19. García-Saenz, J. A., Ayllón, P., Laig, M., Acosta-Eyzaguirre, D., García-Esquinas, M., Montes, M., … Romero, A. (2017). Tumor burden monitoring using cell-free tumor DNA could be limited by tumor heterogeneity in advanced breast cancer and should be evaluated together with radiographic imaging. *BMC Cancer*, 17, 210. doi:10.1186/s12885-017-3185-9

20. Dawson, S., Tsui, D., Murtaza, M., Biggs, H., Rueda, O. M., Chin, S., & Dunning, M. J. (2013, March 28). Analysis of Circulating Tumor DNA to Monitor Metastatic Breast Cancer. *New England Journal of Medicine*, 368, 1199-1209. doi:10.1056/NEJMoa1213261