

**Genomic Heterogeneity of Glioblastoma: A Comparison of the Enhancing
Tumor Core and the Brain Around the Tumor**

A thesis submitted to the University of Arizona College of Medicine – Phoenix
in partial fulfillment of the requirements for the Degree of Doctor of Medicine

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DEDICATION

Dedicated in the memory of my loving grandmother, Wanda Ford. She is one of many who lost their life too young due to GBM. I would like to thank Dr. Tran and his colleagues for the opportunity to work with them on a project that carries a heavy sentimental value for myself. May this R21 study, TGen's future studies, and to all researching GBM significantly impact patient care and provide a longer, meaningful life for patients/family suffering from GBM.

ACKNOWLEDGEMENTS

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Dr. Nhan Tran received his undergraduate degree in microbiology from the University of Arizona and continued on to receive his doctorate in cancer biology there as well. He has 70+ publications, many of which were publications focused on gliomas. At TGen his focus has been on the highly aggressive astrocytoma called Glioblastoma Multiforme. In particular, his research is on the biochemical and molecular mechanisms specific to the genes that the tumor expresses and the GBM's abnormal signaling that can hopefully be specifically targeted for therapeutic treatment. These genes allow the GBM cells to migrate, invade, or induce survival signals. One of the biochemical findings by Dr. Tran includes the up-regulation of a transmembrane protein receptor Fn14 in GBM in comparison to normal brain tissue⁴¹⁻⁴³. This is believed to allow invasion and cell survival; therefore, it is hopeful with further understanding that it can be targeted to create a therapeutic treatment. Dr. Tran has already discovered gene expressions that vary in GBM vs. normal brain matter that can be targeted, however, his focus is also on successfully identifying these targets to yield a patient specific treatment⁴¹⁻⁴⁵. Dr. Tran recognizes the importance of clinical doctors needing to work alongside biological doctors to further medicine that will benefit patients in the most effective, minimally invasive, and patient specific route.

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Project Performance Site/Environment:

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The portion of research that TGEN will conduct will be performed at TGEN in the Brain Tumor Unit (BTU) laboratory. TGEN will be given de-identified slides of human BAT tissue to analyze.

Other Performance Sites:

Image processing and analytics at Mayo Clinic Arizona^{12-14, 22-26}; image-guided stereotactic surgery at Barrow Neurological Institute and Mayo Clinic Arizona²²⁻²⁵; development of Machine Learning (ML) methodology at Arizona State University²¹; cell biology and genetics of tumor within BAT at TGen, University of Minnesota, Mayo Clinic Rochester, and Mayo Clinic Arizona^{4, 26-28}.

ABSTRACT

Glioblastoma Multiforme (GBM) is a highly aggressive brain tumor and comprised of heterogeneous genetic profiles. The genomic diversity seen in GBM makes it difficult to target treatment because of non-uniform responses and the recurrence of the tumor after treatment.¹⁻³

A current obstacle with using conventional MRI to guide a surgical biopsy and resection is the primary focus being on the enhancing tumor core, leaving behind brain around the tumor (BAT). BAT is rarely biopsied, but is known to contribute to a recurrence of the tumor⁴⁻⁶. BAT areas will be the study's focus of genomics. This project is a smaller portion of a large R21 grant, an NIH-funded project, with an overarching goal to determine the genomic expression profiling of BAT and core areas of GBMs, the correlation of MRI imaging to areas of likely recurrence and thereby be able to provide specialized patient treatment by providing a set of image-based criteria (phenotypically) that predict the genomic signatures of treatment resistance.

The experiment conducted in this sub-project, identified genomic characteristics of GBM specimens from the BAT and core regions. TGEN received the biopsied BAT areas of GBM tissue samples, isolated the DNA, performed the array-based Comparative Genomic Hybridization (aCGH), and analyzed the data received from the aCGH. The primary goal is to assess the differences in the genotypes of tumor cells within brain around tumor (BAT) and the cells residing in the core of the tumor. We hypothesized: (1) The BAT genotype will be different from the core of the tumor (2) and we will be able to identify what genomic alterations may explain for the variants/recurrent tumor. Anticipated results were expected to demonstrate genetic heterogeneity within a patient's GBM comprised of more than one, as well as, genomic differences in the brain tumor core compared to the BAT³⁶.

Within the constraint of this sub-project, I was able to complete the genomic analysis of the tumors from three GBM patients. Considering the small sample size, I focused on the genomic analysis of a single patient that yielded the best results via aCGH, Patient 8. Our results demonstrated GBM heterogeneity, biopsy among the spatial location of the tumors to be important, specifically with BAT samples showing only amplification of chromosomal regions

harboring genes implicated in cell invasion and migration via the Wnt/Beta-catenin, CKLF and NFkB pathways.

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INTRODUCTION/SIGNIFICANCE

Astrocytomas, oligodendrogliomas, and ependymomas are the most common primary brain tumors and fall under the category of gliomas⁴⁷. Glioblastoma (GBM) is a diffuse grade IV astrocytoma with poorly defined borders that infiltrate neighboring brain tissue and alter it⁴⁷. This tumor varies in genetic makeup from region to region which is distinguishable from the other regions^{1,2,4}. Upon histology the tumor shows necrosis, hypercellularity, and marked angiogenesis surrounding the tumor⁴⁷.

Figure 1. Gross Anatomy, MRI imaging, and Histology Demonstrating Invasive GBM

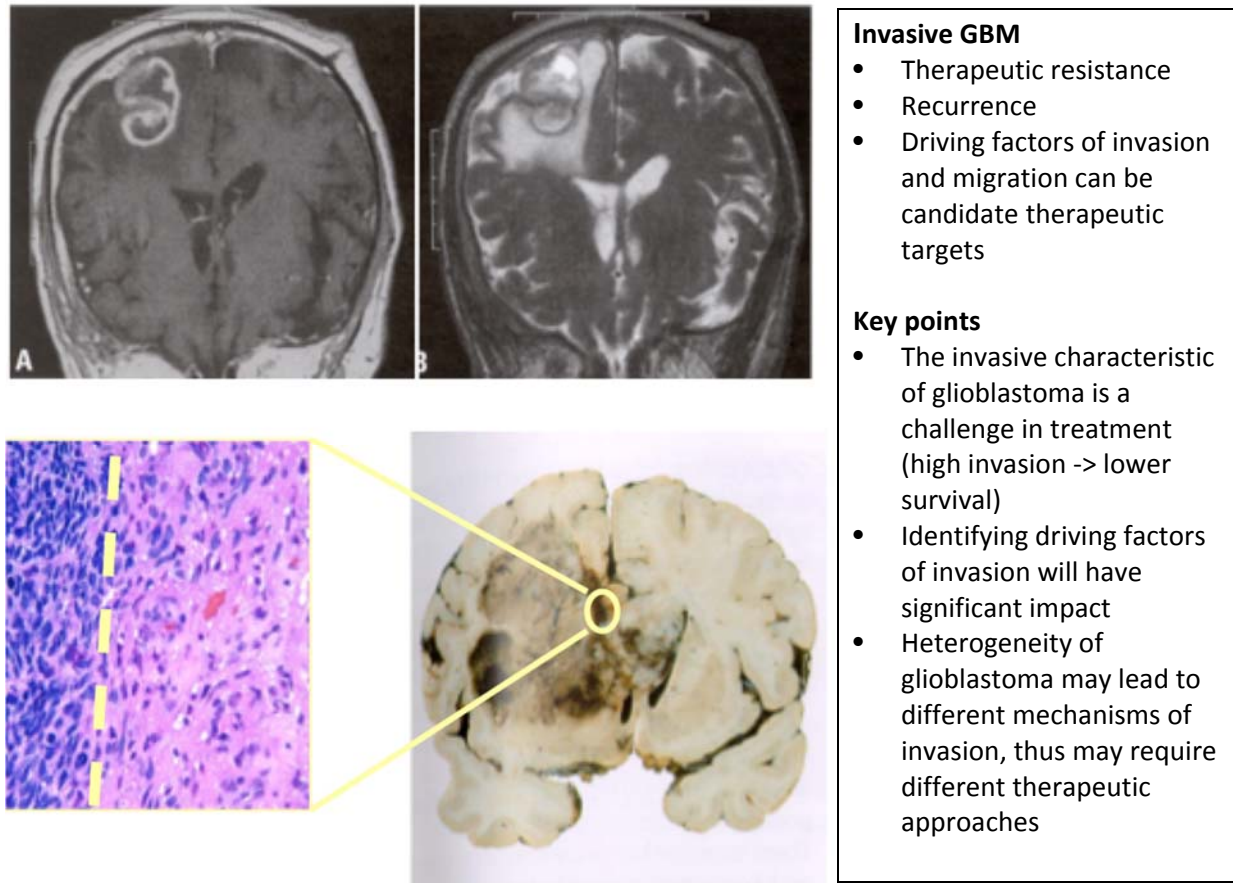
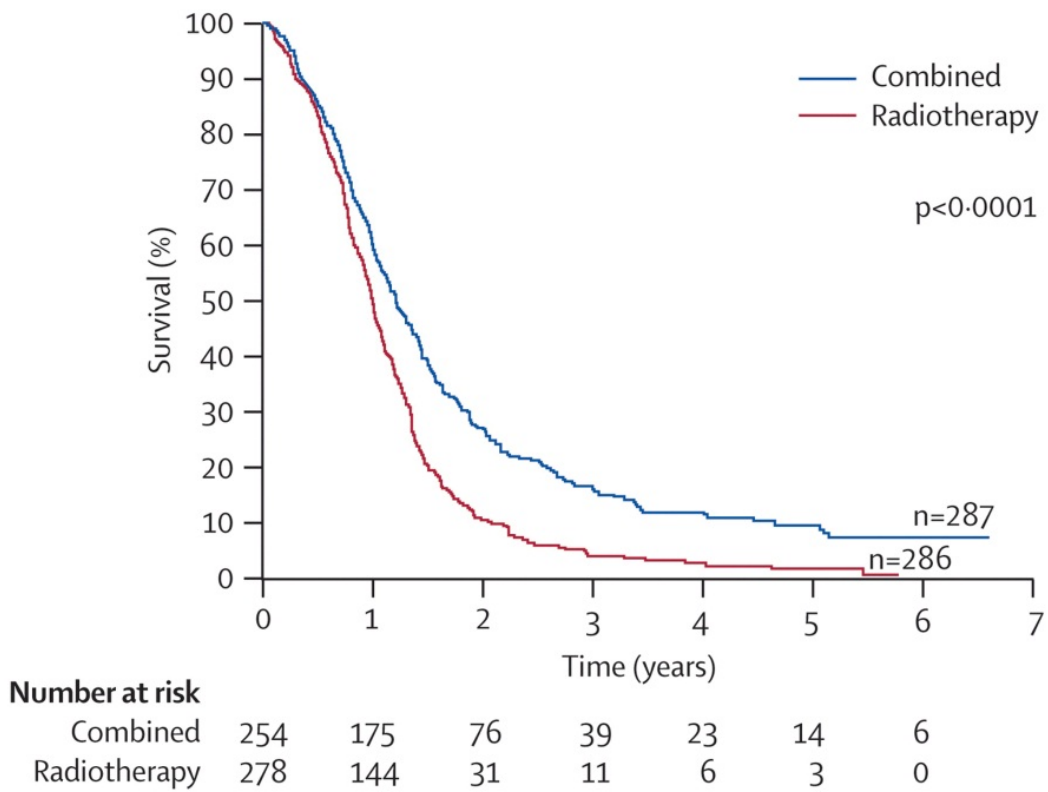


Figure 1. Top left: Coronal MRI that is T1 C+ image demonstrating a supratentorial GBM in the right frontal lobe. **Top right:** Coronal MRI T2 weighted image also demonstrating a supratentorial GBM in the right frontal lobe with surrounding vasogenic edema. **Bottom Left:** Histological representation of a GBM demonstrating hypercellularity, hyperchromatism, and pleomorphic to the left of the yellow line. To the right of the yellow line are areas of vascularity, areas of necrosis, and neoplastic cells palisading around the necrotic area. **Bottom Right:** Gross appearance of a large, diffusely infiltrating, and necrotic mass.

Glioblastoma comprises around 40% of primary cerebral neoplasms seen in the later decades of life. It is the most common intracranial brain tumor in adults and is an aggressive, heterogeneous malignancy with a short life expectancy⁴⁸. After aggressive surgical resection and chemo/radiation therapy, the average life span is only 14 months².

Figure 2. Therapy for Newly Diagnosed GFM: Room to Improve



M. Preusser *et. al.* Ann Neurol 2011; 70:9-21

Figure 2. Demonstrates: Concomitant and adjuvant TMZ: median survival 12.1 → 14.6 months.

The heterogeneity of the composition of this tumor (also seen in other cancers), makes it difficult to treat, whether it is due to the cancer stem cells' (CSC) unlimited ability to proliferate, their resistance to chemo/radiation therapy or the microenvironment of which it can infiltrate and alter^{1,34}. Additionally there is an inability to distinguish the tumor from inflamed normal brain tissue and tissue sampling of the subclonal populations^{5-9,36}. The difficulty with the tissue samples is that GBM is a heterogeneous tumor; therefore, the tissue sample may not be representative of the tumor as a whole or the portion of the tumor directly abutting it. Currently, practiced surgical methods do not project the phenotypic variations of the tumor well, forcing resection of the tumor's core but leaving the recurrent rim⁴⁻⁶. This leaves behind brain around tumor (BAT) that is resistant to therapy and the source of the recurrence⁵⁻⁹. Hence, this area will likely lead to future therapy targeting. Genetically characterizing BAT through new, innovative measures is vital for the future treatment of GBM. The portion of the study that I participated in with TGEN relates to the heterogeneity seen in cancers, particularly GBM. Therefore, we assessed the differences in the genotypes of cells within brain around tumor (BAT) and the core of the tumor. We hypothesized: (1) The BAT genotype would be different from the core of the tumor (2) and we would be able to identify what genomic alterations may explain for the variants/recurrent tumor.

Specific Aim:

Conduct an array-based Comparative Genomic Hybridization (aCGH) of the brain tissue specimens and assess the differences in the genotypes of cells within brain around tumor (BAT) and the core of the tumor. We hypothesize: (1) The BAT genotype will be different from the core of the tumor (2) and we will be able to identify what genomic alterations may explain for the variants/recurrent tumor.

Rationale:

Medicine overall is moving towards patient personalized treatment based on genomics. This is likely realistic for treatment of GBM patients as well. This study will give insight to better understanding current known genetic pathways and the discovery of new key genetic pathways that have potential to be exploited as personalized therapeutic targets^{32, 37}. In the future this could increase patient survival by lowering rates of treatment resistance and tumor recurrence.

Impact:

GBM is a heterogeneous malignant cancer that receives uniform therapeutic treatment. Current research shows genetic abnormalities between GBM vs. normal brain tissue¹⁰⁻¹³. Therefore, different GBM's should be treated differently, keeping in mind that each patient has a different composition of his/her GBM tumor as well³¹⁻³³. While much of the current treatment consists of resecting the core of the GBM, this approach is futile in that the tumor is always recurrent⁴⁷. This study focused on the primary cause of treatment failure in the BAT from where the recurrent tumor stems⁴⁶. For patients, this has the potential to affect how GBM patients are diagnosed and treated based upon genetic analysis of the poorly understood BAT regions; if we are able to augment the discovery of genetic pathways, hence, the patients' potential therapeutic targets. Potentially, this could yield a paradigm shift from a uniform treatment of a non-uniform brain tumor to a multi-selective therapeutic treatment based on the composition of the patient's GBM.

METHODS/MATERIALS

Preliminary studies:

Array-based Comparative Genomic Hybridization (aCGH), DNA Analytics, and GeneSpring, are techniques commonly employed by Dr. Tran's lab and TGEN's bioinformatics team at TGEN⁴¹⁻⁴⁵.

Study Design:

Twelve GBM patients were enrolled by St. Joseph's IRB approved protocol prior to their surgical resection for pre-operative multi-parametric MRI. Due to time and specimen constraint of this smaller sub-project, I was only able to complete the analyses of the GBM tumors from three patients. Stereotactic multi-parametric MRI image guided biopsies are available for each biopsy from the BAT region, and the other biopsies from the core. The final number of biopsies was determined by the neurosurgeon during surgery, particularly the number of BAT biopsies. Multi-parametric MRI helped identify non-enhancing tumor with BAT for sampling. A neuropathologist checked for tissue quality and tumor cellularity of each specimen.

TGEN acquired the samples from St. Joseph's and did not have access to the master list, making this a retrospective cohort case study. The tumor DNA was isolated from multiple frozen biopsies of the three patients and Agilent® SurePrint G3 Human High-Resolution Discovery Microarray Kit was used to determine CNV (Copy Number Variations)^{8, 9, 15}. The control for the aCGH is Agilent's® commercially available pooled reference DNA. CNV were calculated using log change relative to an internal reference. CNV were conducted using software that included DNA Analytics and GeneSpring GX9. Genes that were of special interest were genes that are thought already to be associated as oncogenes. Examples are: angiogenesis (VEGF/VEGFR, PTEN, EGFR), necrosis (bcl2), proliferation (Rb, p53, MAPK, EGFR, mTOR), invasion (src, MMP/TIMP), and vessel permeability (Occludin, Claudin 5)^{4,31}.

Data Analysis:

TGEN analyzed the data of the aCGH and assessed the CNV that appeared to be aberrant. Hierarchical clustering was used to show relations between samples based on similarity³⁹⁻⁴⁰. We conducted genetic pathway analysis, looking for a driver of GBM^{39-40, 46}. Agilent® Oligonucleotide Array-Based CGH for Genomic DNA analysis from DNA isolation to feature extraction of the array data onto our genomic workbench version 7 was used for analysis. We analyzed the array data in Genomic Workbench 7.0 for Copy Number Variations (CNV).

Expected Outcomes:

We expected to see heterogeneity within each GBM patient and across the cohort, a genomic difference of CNV between BAT vs. core tumor, and verify certain known CNV that correlate with others or discover patterns that are currently unknown between CNV.

Risks/Resolutions:

There are no direct patient risks in TGEN's portion of the research. The foreseen leading downfall was human error, making a specimen insufficient for further use in the study. Another potential pitfall included normal tissue being present in BAT specimens. BAT biopsies are retrieved along the rim of the GBM; consequently, these specimens contained normal and malignant brain parenchyma. Normal brain parenchyma dampens the signals of aberrations, thereby potentially making the aberrations of malignant brain parenchyma quiescent in analysis. Therefore, for BAT specimens the threshold was lowered during analysis. The sample size of n=3 is small. Unfortunately, it is the amount we were able to obtain due to time constraint.

We derived solutions and methods to trouble shoot for potential foreseen obstacles/errors: 1.) When nanodropping if we found a low 260/230 or 260/280 value that indicated contaminants, we would repurify per the DNeasy protocol⁴⁹. 2.) Poor sample quality due to degradation for non-FFPE samples on the gel with no high-molecular weight band but a trail of DNA to the low molecular weight band, which would mean the DNA was degraded. We would have checked the DNA by running a gel electrophoresis, then repurified⁴⁹. 3.) If the

estimated concentration of DNA was too high or low, re-quantitated it to make sure quantitation was accurate and make sure to not use different isolation methods for our samples and reference⁴⁹. 4.) Low degree of labeling that was not due to poor sample quality could have resulted from sub-optimal labeling conditions such as too many freeze-thaw cycles of the Cyanine dUTP, enzyme degradation from being warm for too long, volume mistakes, or over-exposure to light and/or air⁴⁹. 5.) Post-labeling signal loss could have been due to conditions that were too stringent so the Cyanine 5 signal was degraded⁴⁹. This could have been due to exposure to the ozone, pollution, compressors, and centrifuges. If this were to have occurred, we would have tried to find the cause and adjust accordingly. 6.) High BGNoise could have lowered signal-to-noise values and increased DLRSD values⁴⁹. BGNoise is defined as the standard deviation of the signals on the negative controls. If this were to have occurred, we would have checked the microarray image for visible non-uniformities because high BGNoise is often introduced during the washes. We would have also made sure the dishes, racks, and stir bars were clean with either MilliQ water or if necessary acetonitrile wash.

Alternative Strategies:

If aCGH did not suffice, then NextGEN sequencing could have been used to complete DNA sequencing and whole genome sequencing. FISH analysis could have been used to validate regions of gains/losses within a single sample².

RESULTS

Described below are the data from the analyzed specimens of three different patients through an array aCGH that I performed. The number of BAT and enhancing core tumor (enh) biopsies that were analyzed are given:

- Patient 2: 3 samples (2 Enh, 1 BAT)
- Patient 4: 5 samples (3 Enh, 2 BAT)
- Patient 8: 14 samples (7 Enh, 7 BAT)

Considering the small sample size, I focused on analyzing a single patient, Patient 8. Patient 8 yielded the best results of the three patients to analyze, due to sample quality and number of biopsies. In total, 14 specimens from patient 8 were acquired and 7 were BAT, 7 were enh core, and 1 marginal (not included in analysis). Two of the BAT samples were determined inadequate to the neuropathologist, meaning there was predominately too much normal brain parenchyma. Two enh core biopsies were excluded from analysis to balance BAT and enh samples; otherwise, the results would have been falsely, drastically skewed.

PATIENT 8 RESULTS

Figure 3. MRI Images of Patient 8’s GBM, Locations of Biopsies, and Type of Tissue Retrieved.

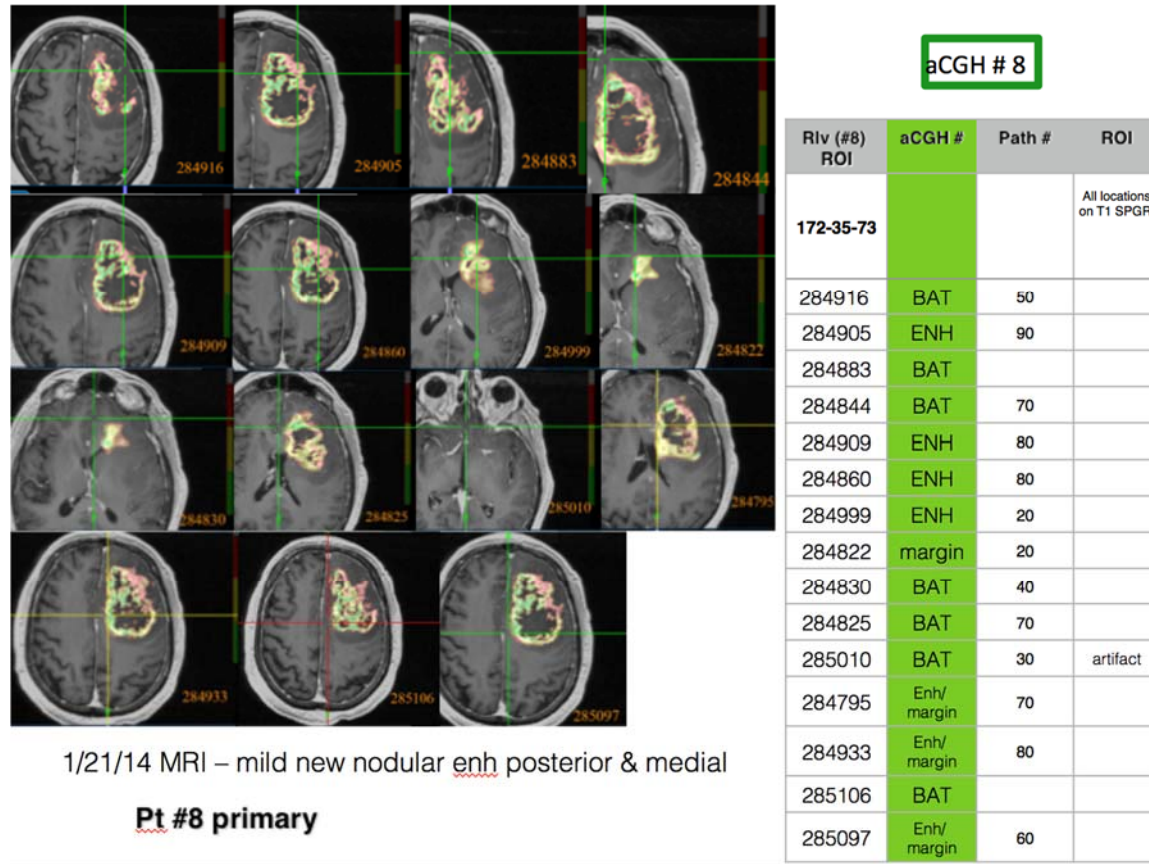


Figure 3 MRI images of a left frontal lobe GBM with the various biopsy locations depicted by the intersection of the green lines. Table: ROI (regions of interest) column are the biopsy locations coded to correlate with the MRI that will be sent to ASU to incorporate into their machine learning algorithm, aCGH column identifies if the sample is BAT or enh, and the path column displays percent tumor of the sample.

Figure 4. Displaying a Comprehensive View of our Samples from Patient 8.

Patient 1723573 (#8)

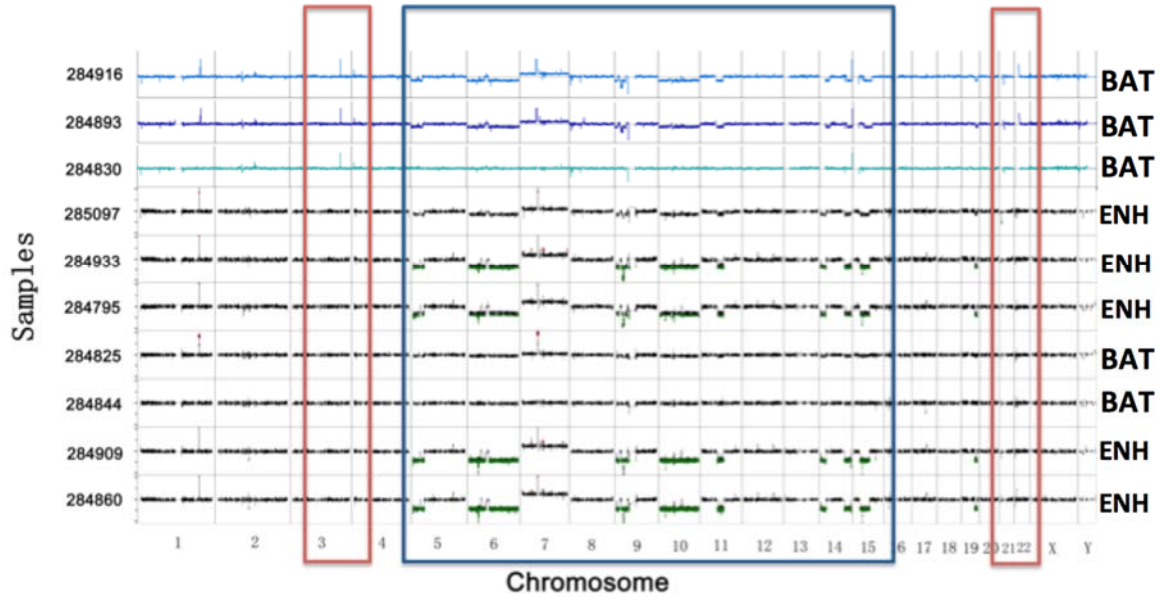


Figure 4. Gross genomic view of Patient 8's samples. Chromosomes 3 and 22 display focal amplifications in BAT samples and Chromosomes 5-15 demonstrate similar changes evident in both BAT and enh samples.

Table 1. Patient 8's BAT Samples CNV Commonalities; Only Amplifications Observed.

GENE SYMBOL	GENE NAME
ARR-19 (also referred to as CMTM2)	Cytoskeleton remodeling of various actin-binding proteins, androgen receptor inhibitor
VE-cadherin	Vascular endothelial cadherin
CKLF	Chemokine-like Factor
CMTM1-4	CKLF-like MARVEL transmembrane domain-containing protein 1-4
DPP-10	Dipeptidyl peptidase
CA7	Carbonic Anhydrase 7
APP-BP1 (also referred to as NAE1)	NEDD8-activating enzyme E1 regulatory subunit
BEAN1	Protein BEAN1/Brain-expressed protein associating Nedd4 homolog
TRAP25 (also referred to as MED30)	Mediator of RNA Polymerase II transcription subunit 30
PDP2	Pyruvate Dehydrogenase Phosphotase 2
DYNC1L12	Cytoplasmic dynein 1 light intermediate chain 2/Dynein light intermediate chain 2, cytosolic
CCDC79	Coiled-coil domain-containing protein 79
TK2	Thymidine kinase 2, mitochondrial
SLC30A8	Zinc transporter 8

Table 1. Summary of Patient 8's CNV amplifications or loss in the BAT samples. Interestingly, only CNV amplifications were observed. The gene symbol and name are given of the amplifications.

DISCUSSION

GBMs arise either *de novo* (>90%, primary GBM) or progress to GBM from lower grade gliomas (~10%, secondary GBM). When one thinks about GBM it is best to take a landscape view starting from 20,000-feet away and where we are readily able to identify an amplification of chromosome 7 and a deletion of chromosome 10. Subsequently, leading to EGFR gene amplification on chr 7 and loss of PTEN on chr 10, respectively. Although GBM is heterogeneous when comparing different portions like enh and BAT, there is no major rearrangement seen in this chr 7 and 10 pattern throughout most GBM patients.

If one goes from a 20,000-foot view to an up-close view, we can begin to appreciate on the minute and subtle changes. These minor, almost quiet genetic changes can potentially be the inciting alteration leading to invasion and migration observed in BAT. Common to all three patients, hence observed in patient 8's samples, is amplification of genes involved in the Wnt and NFkB pathway. Somatic changes/mutations signify permanent changes in DNA; whereas, epigenetic changes result in subtle changes (not mutations) that may modify gene expression. Genome alterations hold an important role in explaining cell invasion and migration.

Looking at specific commonalities and/or differences in the BAT samples of patient 8, only gene gains were found when comparing BAT samples. Again to focus on these similarities listed in Table 1, we can derive a story:

Arr-19 inhibits androgen receptor (AR)⁵⁰. AR is thought to compete with Wnt signaling by way of beta-catenin⁵¹. Therefore, if you block AR it yields more beta-catenin for the Wnt canonical pathway⁵¹. The increased levels of Wnt canonical pathway beta-catenins are able to escape ubiquitous degradation and can translocate into the nucleus to undergo transcription. Beta-catenin also binds to VE-cadherin, aiding in the process of invasion into the vascular endothelial⁵². Beta-catenin binds to VE cadherins, allowing the cadherins to bind together at the cytoskeleton junction⁵³. As the endothelial cells contract, these newly formed cell-cell adhesions will be the root of creating a permeable barrier⁵⁴. CKLF, as well as being involved in chemotaxis (migration), acts as actin polymerization⁵⁵. CKLF and CMTM1-4 are essential agents involved in tumor and stromal chemotaxis during progression and metastasis of tumor

dissemination⁵⁶. The more readily available actin and beta-catenin are, the more gaps that can be formed, thus, paving the way for invasion to occur under chemotaxic guidance.

Many proteins undergo ubiquitination via the Neddylation pathway; neither NFkB nor its inhibitor are exempted from this inevitable fate. NFkB's inhibitor is bound to it⁵⁷. When NFkB and its inhibitor go through Neddylation, NFkB is freed from its inhibitor; allowing NFkB to be involved in cell survival, invasion/metastases (NFkB turns on transcription of VE-Cadherin), and inflammation⁵⁷⁻⁶¹. NEDD4 is an E3 ubiquitin-protein ligase that transfers ubiquitin to the ubiquitin-targeted substrate marking it for imminent degradation⁶²⁻⁶³. BEAN1 binds to the WW domains of NEDD4 and an increase in both proteins leads to an escalation in neddylation, indirectly increasing NFkB activity, which promotes cell migration⁶²⁻⁶⁴.

Another appreciable amplification alludes to migration/metastasis. Studies have shown that mRNA of TRAP25 isoform is present only in circulating cells, but is not expressed in cultured adherent cells⁶⁵.

One clear observation is that BAT gene expression is amplified to favor invasion and migration. Although we were not able to elucidate a driver, we can appreciate the gain in gene expression of BAT tissue to favor invasion and metastasis in the epithelial-mesenchymal transition.

FUTURE DIRECTIONS

With the current imaging, it is difficult to distinguish the BAT from the core tumor or the inflammation due to treatment²⁹⁻³⁰. Some studies have already successfully demonstrated selected varying phenotypes within BAT by non-invasive measures^{10-14, 29-30}. The various techniques listed are: Diffusion Weighted Imaging (DWI), Diffusion Tensor Imaging (DTI), perfusion MRI (pMRI), Dynamic Contrast Enhanced (DCE-MRI), T1Weighted (T1W) and T2Weighted (T2W) Conventional MRI^{10-14, 29-30}. The tumor phenotype is as follows respectively: Cell density/proliferation, tumoral white matter infiltration, angiogenesis, vascular permeability, hypoxia/necrosis (T1W), and tissue water content (T2W)^{10-14,29-30}. These studies focus on specific gene loci and specific imaging techniques. For GBM that exemplifies such a dynamic, robust genetic compilation, it is of the best interest to expand analysis via incorporation of multiple complementary MRI techniques to get an appropriate representation of its genomic diversity^{3,38}.

By integrating genomic data and multi-parametric MRI, extending our sparse ML (Machine Learning) methodology, we could be able to better characterize the regional genomic variations in BAT. Developing these correlations would hopefully yield MRI's predictive accuracy and greatly influence how GBM patients are diagnosed and treated. Therefore, in the future MRI mapping may be able distinguish genetically different regions of BAT (of those shown to have prognostic significance); and that in conjunction with ML analysis, can help demonstrate genetic profiles and subclasses throughout the GBM core and BAT. For the patient, this has the potential to affect how GBM patients are diagnosed and treated based upon this innovative methodology in the poorly understood BAT regions³¹⁻³⁴.

Furthermore, we could correlate the genomic profiles of BAT with local treatment response and tumor progression. Potentially yielding a novel perfusion MRI method, which distinguishes regional tumor growth from radiation injury^{22,37,38}.

However, it is imperative that we continue to strive to discover the driver of invasive GBM, which would open a floodgate of information pivotal in patient care and treatment. The system could augment the discovery of genetic pathways; hence, potential therapeutic targets⁴⁶. This could yield a paradigm shift from a uniform treatment of a non-uniform brain

tumor to a multi-selective therapeutic treatment based on the composition of the patient's GBM; affectively targeting, treating, and monitoring by way of personalized medicine.

CONCLUSIONS

GBM is the most common primary central nervous system tumor carrying a poor prognosis. As mentioned above, invasive GBM is currently resistant to therapy, and is the cause of recurrence.

Our results demonstrated GBM heterogeneity and importance of the biopsy among the spatial location of the tumors, specifically with BAT samples. An increase in amplification of genes involving the Wnt/B-catenin, CKLF and NFκB pathways were observed; which promote invasion and migration, also known as, mesenchymal-endothelial transition. Currently, a biopsy can miss the portion of the tumor that is not susceptible to the specific treatment, especially in regions of BAT. Which are regions rather difficult to visualize during surgery. Subsequently, this portion continues to grow uninhibited. As such, the future direction of this study is aimed to non-invasively identify the genotypic profile of patients GBM phenotypically via MRI. This is of profound importance in patient specific care, treatment, and outcome.

Heterogeneity and the invasive characteristics of glioblastoma are challenging in treatment resulting in low survival rates. The heterogeneity of GBMs also leads to different mechanisms of invasion, thus, will likely require different therapeutic approaches. Further analysis is needed to determine the genetic drivers of invasion, migration, and survival in GBM as these are probable therapeutic targets; thus, could have significant, translational impact for individualized patient care.

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