Background and Significance

Glioblastomas (GBM) occur as the most prevalent and lethal malignant tumor-bearing cancer in adult brains. While 60% of GBMs are observed in the four lobes of the brain (frontal, temporal, parietal, and occipital), they also may occur in the cerebellum, brain stem and spinal cord. The median age of glioblastoma formation is 64 years, however, they can arise from childhood onward (1). Majority of glioblastomas are considered as primary (de novo), while some are classified as secondary where an existing tumor transforms into a GBM over time. Therapeutic treatments such as chemotherapy and radiation have been beneficial to some patients, but despite these treatments survival time is projected at only 14.6 months (2).

The Cancer Genome Atlas Project revealed the genetic profile for glioblastoma cells and identified three significant signaling pathways activated in these cells, where GBMs experience changes affecting cell proliferation and survival. These pathways include: the p53 pathway, tyrosine kinase/Ras/phosphoinositide 3-kinase signaling pathway, and the retinoblastoma pathway (2). Genetic mutations and gene expression patterns observed between primary and secondary GBM cells differ; where primary GBMs experience EGFR overexpression, PTEN mutations, and chromosome 10q loss, and in secondary GBMs IDH1 mutations, p53 mutations and chromosome 19q loss are observed (2, 3).

Considering the known signaling pathways and expected genetic alterations observed in primary and secondary glioblastomas, targeted therapeutic treatments may be developed to elongate patient survival, and reduce tumor cell proliferation. Combination chemotherapy treatments were performed on 4 patients (now deceased) with glioblastomas, and samples were collected pre-treatment and at each timepoint of relapse. To discover why the treatments failed, three experiments will be performed on these samples, including RNA-seq to analyze the transcriptome, next-gene DNA sequencing to identify mutations, and genome-wide sequencing to explore epigenetic trends.

Experiment 1

Rationale

The first experiment would be to perform whole-genome DNA sequencing (WGS) on samples from each time point to determine genome-wide mutation signatures, subclonal mutations, and mutations across the genome. Glioblastoma samples from four patients at three timepoints from here will be considered as follows: T1 is the early timepoint before combination chemotherapy treatment, T2 is the timepoint after the first treatment relapse, and T3 is the final timepoint after another relapse. It is important to sequence and analyze samples at each time point to determine significant mutations or modifications after treatment and relapse. These mutations may play a role in uncontrolled cell proliferation and tumor survival, and could be therapeutic targets for future treatments.

Methods

Genomic DNA from samples at each timepoint T1, T2, and T3 will be extracted and fragmented for library preparation, amplification, and sequencing. Samples will be then run through high-throughput next-gen sequencing for WGS (ie. Illumina sequencing). Resulting raw data will be put through quality control (QC analysis), and analyzed for identification of mutations across all timepoints in comparison to a reference genome.

Results

Expected results from this experiment include the identification of significant mutations and genomic modifications across all timepoints (deletions/insertions/SNPs). Identification of these changes between treatment and relapse could shed light on genetic associations with treatment resistance, as well as to highlight potential mutation targets for future therapeutics. Some known mutations associated with glioblastoma proliferation, treatment, and relapse include: IDH1 (isocitrate dehydrogenase 1) mutation, which results in the gain of function

to catalyze hydroxyglutarate function, leading to tumor development (4). Other mutations observed in glioblastoma samples include SNPs in EGFR, deletions in NF1 (inhibitory tumor-suppressor gene for BRAF and RAS genes), and PIK3CA and PIK3R1 genes (4). All of these genes are involved in tumor suppression and regulation of cell proliferation, so mutations in these genes in glioblastoma samples result in uncontrolled proliferation of tumor-bearing cells.

Challenges

Some challenges that could arise from performing whole genome sequencing include the high computational requirement. While it is not the most monetary and computationally cost-effective method to use, it allows for an in-depth look at potential genetic modifications that could contribute to relapse. Additionally, the comparison of samples to a reference genome may not account for genetic variation associated with the patient's demographic. To resolve this, targeted regions that are known to be associated with glioblastoma function, proliferation, and potential mutations should be assessed first. CBioPortal is used to identify the top 50 expressed genes in glioblastoma cells, and can be used alongside these experimental approaches to identify mutations, biomarkers, or potential targets for therapeutics.

Experiment 2

Rationale

The second approach will be to analyze the transcriptomes for samples from all three timepoints to identify differentially expressed genes and potential pathways of significance. By identifying changes in gene expression between samples, we may be able to observe mechanisms that contribute to treatment resistance and ultimate relapse. Additionally, performing RNA-seq on our samples could provide insights into biomarkers associated with cell survival, providing a better understanding of the expected prognosis and further development of therapeutic treatments. To ensure that there is enough sample to last, we can grow a portion of each in mammalian cell culture and continue observation as cells continue to proliferate.

Methods

From glioblastoma samples at each timepoint, mRNA would be extracted and purity checked on a gel before fragmentation and cDNA generation via reverse transcription. In reverse transcription, a dT oligo primer would hybridize to the 3' poly-A tail on the mRNA fragments, while the reverse transcriptase enzyme reverse transcribes the fragments into single stranded cDNA. Using (real time) rt-PCR with RNase, DNA polymearase, and DNA ligase, a library of double stranded cDNA fragments can be generated. This library can then be used in high-throughput sequencing, with Illumina sequencing by synthesis. Sequence results will be analyzed in comparison to the reference genome, and DEGs (differentially expressed genes), biomarkers, and significant pathways can be identified. Enrichment analysis can then be performed to confirm these significant DEGs and the association with function and phenotype in glioblastomas.

Results

Sequencing results would produce millions of short reads, which will be sent through quality control (QC) and sequence processing using R studio, and subsequently aligned to the reference genome. This comparison to the reference, and comparison between T1, T2, and T3 samples should highlight differential gene expression, potential biomarkers, and further understanding for pathway analysis. The confirmation of significant DEGs using enrichment analysis may confirm known genetic alterations, as well as uncover modifications between timepoints that may have contributed to the relapses between treatments. Expected molecular biomarkers to be found from performing RNA-seq on glioblastoma samples include p53, RTK/RAS/PI3, RB, MDM2, PI3K, and CDK4, where CTLA-4 is a potential biomarker associated with treatment response (5).

Challenges

Some challenges that may arise in using RNA-seq could be biases during library preparation, which could be resolved by normalizing the data during this process. Another challenge could be the small sample group of 4 patients, which would allow for more variation in the dataset and more difficult statistical analysis of the dataset. Batch effects may also prove to be a challenge to this experimental approach, where unwanted variation may occur during library prep. To avoid this, samples could be randomized, or a healthy negative control sample could be introduced when processing the data for comparison.

Experiment 3

Rationale

The third experiment will utilize ChIP-seq to identify epigenetic changes across the sample timepoints. We can also observe histone modifications, DNA methylation, and potential promotors or transcription factor binding sites, which would give insight into potential therapeutic targets. Identification of DNA methylation profiling has led to higher success than transcriptomes in prognostic prediction of glioblastoma patient survival (6).

Methods

To perform ChIP-seq, or chromatin immunoprecipitation and sequencing, chromatin must be extracted from the samples T1, T2, and T3 from all 4 patients. Immunoprecipitation will then be performed on the DNA fragments using antibodies specific to targeted histone modifications. The immunoprecipitated sample will be sequenced to identify patterns of these histone modifications.

Results

Expected results include identification of target histone modifications, which would uncover regulations by different sets of transcription factors, as well as methylation profiling and active promoter identification. This comparison of epigenetic changes to gene expression patterns can give insight into regulation of gene expression in glioblastomas. Observation of DNA methylation patterns could highlight promoter regions, tumor suppressor genes and oncogenes involved in glioblastoma proliferation. One study showed that transcription factor motifs revealed epigientic regulation of ATAC50 clusters in glioblastoma cells (6). Understanding these changes could lead to further development of targeted therapeutics and better prognosis post-treatment.

Challenges

Challenges may arise that are associated with the antibody specificity, which could be confirmed via western blot prior to immunoprecipitation to confirm correct detection of the target. Another issue that could arise is in the small sample group of four patients, where variability between patients may be high. In this case, the dataset may not show consistent results of epigenetic changes throughout each timepoint.

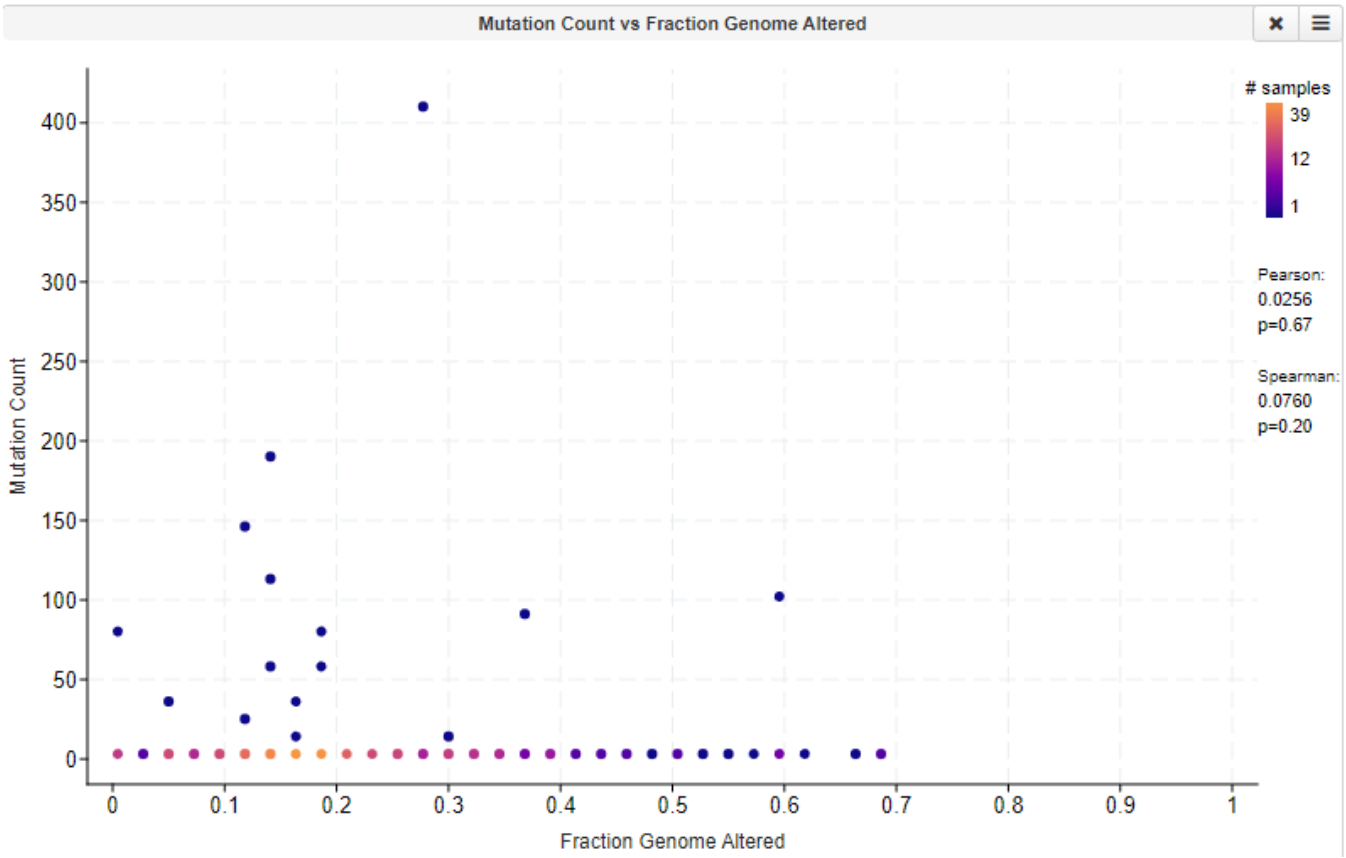
Interpretation

The results from all three of these experiments on glioblastoma samples of four patients before treatment, after first treatment and relapse, and after final treatment and relapse, allows us to further understand the genetic, transcriptomic, and epigenetic changes between timepoints. Contributions to the post-chemotherapy relapses could be observed in genetic resistance to chemo therapeutics, as well as mutations of genes and oncogenes to resist cell proliferation suppression. This could be observed in the results from the WGS analysis, where genetic mutations, subclonal mutations, and mutational signatures are identified as significant contributors to continued proliferation of the glioblastomas. Mutations including SNPs in EGFR, deletions in NF1 (inhibitory tumor-suppressor gene for BRAF and RAS genes), and PIK3CA and PIK3R1 genes involved in tumor suppression (4).

To avoid relapse in future patients, it is important to understand the implications of these mutations, which when deactivated via mutation may cause uncontrolled proliferation of malignant glioblastomas. These mutations could be targets for therapeutic treatments in future patients, and it may be necessary to sequence the samples as treatment is performed. The RNA-seq experiment allows us to visualize the transcriptome and identify any differentially expressed genes between patients and between timepoints of treatment and relapse. If these genes change between treatments, this may provide some insight into reasons for treatment resistance and relapse. Additionally, we can observe which pathways are significant in association with glioblastoma generation, proliferation, and survival post-treatment. Finally, the experiment for epigenetic changes can show us histone modifications and DNA methylation between timepoints, which could point out potential promoters or transcription factors associated with glioblastoma survival. The tool cBioPortal was used here as well to identify the top 20 mutated genes associated with glioblastomas, which provides a foundation for areas within the genome and transcriptome to search for significant modifications. Many of these genes are involved in tumor suppression and regulation of cell proliferation, so mutations to them would cause unregulated development and survival of glioblastomas, resulting in relapse. These top 20 genes include: TERT, PTEN, TP53, EGFR, NF1, ATRX, RB1, PIK3CA, PIK3R1, IDH1, PTPN11, STAG2, FAT1, PDGFRA, KMT2D, SETD2, NOTCH3, NOTCH1, FLT4, and ARID1A. These correspond with findings from multiple publications highlighting observed mutations and their effects on tumor suppression in glioblastoma samples.

Figures

Top mutated genes of Glioblastoma multiforme



Top 20 most frequent mutated genes in Glioblastoma multiforme

Mutated Genes (286 profiled samples)

Gene	# Mut	#	Freq
TERT	232	224	78.3%
PTEN	112	108	37.8%
TP53	131	106	37.1%
EGFR	79	60	21.0%
NF1	62	48	16.8%
ATRX	50	46	16.1%
RB1	40	39	13.6%
PIK3CA	40	36	12.6%
PIK3R1	31	29	10.1%
IDH1	22	22	7.7%
PTPN11	23	21	7.3%
STAG2	21	18	6.3%
FAT1	25	17	5.9%
PDGFRA	21	15	5.2%
KMT2D	34	15	5.2%
SETD2	15	14	4.9%
NOTCH3	17	14	4.9%
NOTCH1	19	13	4.5%
FLT4	16	13	4.5%
ARID1A	19	13	4.5%

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