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Article

Contrasting role of *ASCC3* and *ALKBH3* in determining genomic alterations in Glioblastoma Multiforme

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SUMMARY

Glioblastoma Multiforme (GBM) is the most malignant brain tumor with the highest fraction of genome alterations (FGA), manifesting poor disease-free status (DFS) and overall survival (OS). Methylation status of O-6-methylguanine-DNA methyltransferase (*MGMT*) is the most promising biomarker known to have better therapy prediction and prognosis. However, *MGMT*'s predictive abilities are effective in only 50-60% GBM patients, suggesting a need to identify new biomarkers. We explored The Cancer Genome Atlas (TCGA) and cBioportal public dataset-Firehose legacy GBM to study DNA repair genes Activating Signal Cointegrator 1 Complex Subunit 3 (*ASCC3*) and Alpha-Ketoglutarate-Dependent Dioxygenase AlkB Homolog 3 (*ALKBH3*). To test our hypothesis that these genes have correlations with FGA and can better determine prognosis and survival, we sorted the dataset to arrive at 254 patients. Analyzing using RStudio, both *ASCC3* and *ALKBH3* demonstrated hypomethylation in 82.3% and 61.8% of patients, respectively. Interestingly, low mRNA expression was observed in both these genes. We further conducted correlation tests between both methylation and mRNA expression of these genes with FGA. *ASCC3* was found to be negatively correlated, while *ALKBH3* was found to be positively correlated, potentially indicating contrasting dysregulation of these two genes. Prognostic analysis showed the following: *ASCC3* hypomethylation is significant with DFS and high *ASCC3* mRNA expression to be significant with OS, demonstrating *ASCC3*'s potential as disease prediction marker. Further research using *in vitro* studies and mechanistic analysis of *ASCC3* and *ALKBH3* is needed to better understand their roles in causing genetic alterations in GBM.

INTRODUCTION

Glioblastoma Multiforme (GBM) is a grade IV brain tumor most commonly affecting glial cells, the supporting cells of the brain (1). Although GBM is a rare disease, with an incidence of only 3.21 in a group of 100,000 people, it has a low post-diagnosis survival rate of 3–5% for three years (2, 3). This low survival rate has led to a higher demand for more research to improve GBM treatment and survival. The disease also more commonly affects men than women, where men are 1.57 times more likely to be diagnosed with GBM than women (4). GBM occurs *de novo* in more than 80% of adult GBM patients, primarily in those between 40 and 60 years old. There are instances of secondary GBM as well; however, the molecular mechanisms for secondary gliomas may be different. This study focuses on primary GBM (5). Primary GBM is caused by a genetic dysfunction that often affects the way genes produce proteins, which is common in cancer since multiple genes are altered genetically or through epigenetic modifications (6). At the molecular level, this leads to a dysfunctional relationship between DNA methylation and mRNA expression, thereby causing alterations in the genome, measured as the fraction genome altered (FGA) (7). An increase in FGA indicates an increase in uncorrected DNA, combined with the dysfunction of DNA repair genes that has led to a higher presence of mutations in cell replication and growth.

Aberrant epigenetic changes, unlike mutations, are changes to DNA that occur without changing its sequence, hence affecting a protein's expression, regulation, production, and activity (6). It is essential to study epigenetic changes, as they have an important role in therapy prediction and prognosis (6). DNA methylation, the most widely studied epigenetic change, involves the removal or addition of methyl (CH_3) groups to the DNA strand in the promoter region of the gene (8). The methylation may cause a change in gene expression, as the promoter region is where proteins bind for transcription, thereby regulating and impacting the functional activity of genes (8). Abnormal methylation is categorized as hyper- and hypomethylation, where the number of methyl groups on DNA is increased or decreased, respectively (9).

Although a large array of studies on GBM and its genetic mechanisms have been conducted, O-6-methylguanine-DNA methyltransferase (*MGMT*) is the only gene whose methylation status is a well-known predictive and prognostic biomarker for GBM. *MGMT* is prominent, as it is the only gene responsible for the direct reversal of DNA damage and has a key role in early tumorigenesis and in immediate treatment response (10). *MGMT* promoter methylation is the lone significant biomarker for GBM, and therefore looking for additional markers is necessary (11). There are other genes involved in DNA damage reversal pathway for reversing alkylation damage, during which alkyl groups are transferred to DNA. DNA alkylation causes abnormal base pairings and strand breakage that often leads to cell death. Although the genes in the alkylation damage reversal pathway are unlike *MGMT* because they reverse alkylation damage, they are still important in reversing DNA damage in GBM (12).

The DNA damage reversal pathway, consisting of 8 genes, contains sub-pathways of Alpha-Ketoglutarate-Dependent Dioxygenase AlkB Homolog 3 (*ALKBH3*) mediated reversal of alkylation damage, *ALKBH3*-mediated

reversal of DNA damage, and reversal of alkylation damage by DNA dioxygenases, which offers indirect and direct DNA repair (12). *ALKBH3* encodes a protein that protects against methylation cytotoxicity by repairing single-stranded DNA (13). In addition, Activating Signal Cointegrator 1 Complex Subunit 3 (*ASCC3*) encodes a protein belonging to a family of helicases – enzymes involved in the ATP-dependent unwinding of DNA – and is also a key member of the DNA damage reversal gene involved in DNA repair (14). The DNA damage reversal pathway ensures the correction of damaged regions of DNA based on chemical damage before DNA is replicated (15). *ASCC3* and *ALKBH3* are interconnected in the DNA damage reversal pathway (**Figure 1**). The mutation or dysfunction of *ASCC3* and *ALKBH3* has been studied in some disorders and diseases. Biallelic mutations, or mutations that exist in both gene alleles, in *ASCC3* have been found to cause neuromuscular syndromes (16). Meanwhile, *ALKBH3* has been associated with salivary gland carcinoma and prostate cancer (17). However, to the best of our knowledge, in GBM nothing is known about their role or their impact on disease progression and overall survival (OS), the time after treatment the GBM patient survived. Progression of disease is measured as disease-free status (DFS), or the time after primary GBM treatment that a patient remains disease-free. Measuring the impact of the DNA repair on the two survival parameters is an important aspect of studies, as it indicates potential prognosis for patient's life and therefore may lead to discovery of important biomarkers. Factors of age and sex heavily affect patient response to GBM, with older patients' abilities to fight against cancer being lower than younger patients, and males being affected by GBM more often than females (4). We wanted to explore the potential of *ASCC3* and *ALKBH3* as biomarkers for GBM, owing to their functional similarity to *MGMT*, using publicly available online databases. We aim to study the role of methylation and corresponding mRNA expression of *ASCC3* and *ALKBH3* in affecting FGA in GBM patients.

RESULTS

Patient characteristics, methylation, and mRNA expression of *ASCC3* and *ALKBH3*

Data filtering according to our defined inclusion criteria of the clinical and experimental data obtained from cBioportal and The Cancer Genome Atlas (TCGA) led us to our

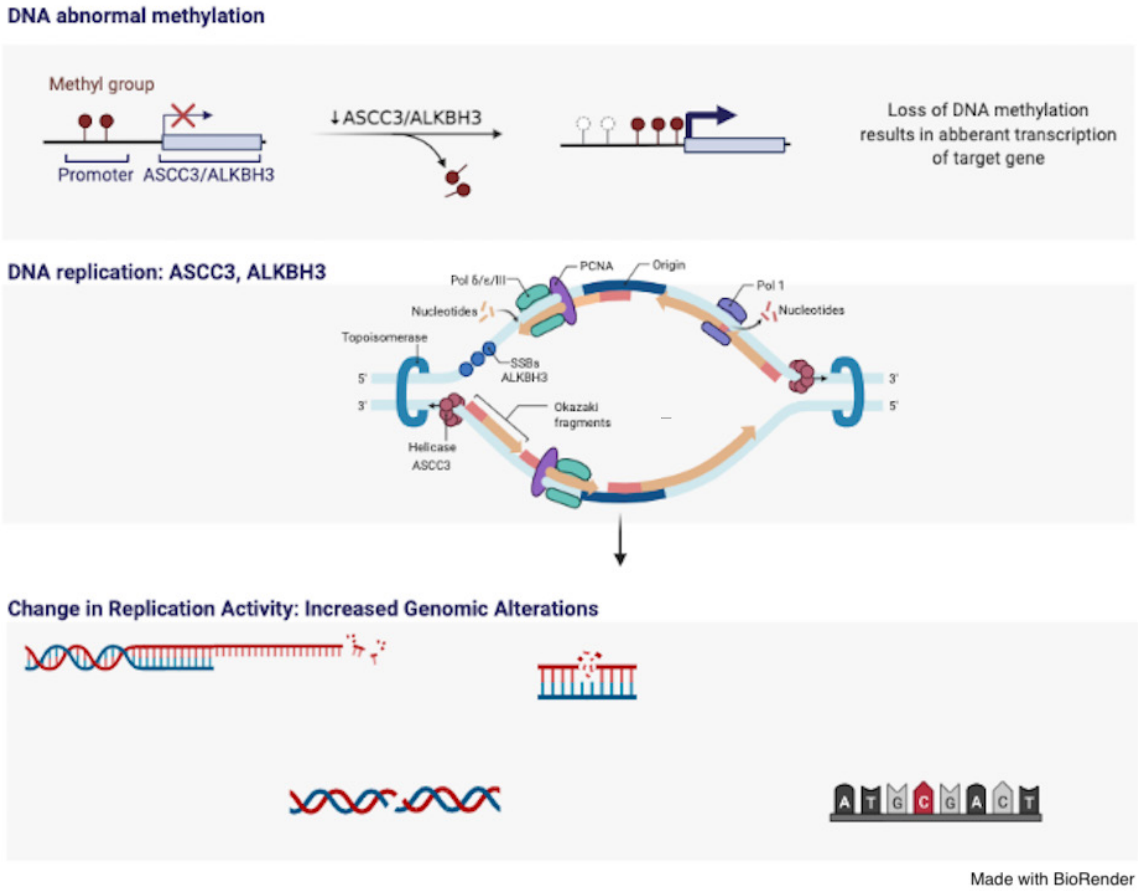

Figure 1: Epigenetic Dysregulation in GBM via abnormal DNA methylation and DNA replication through *ASCC3* and *ALKBH3*. A. The abnormal methylation process in the promoter regions of *ASCC3* and *ALKBH3* is shown through the loss of methyl groups in the region, also known as hypomethylation. **B.** DNA replication via *ASCC3*'s helicase in the double stranded DNA and the ALKBH3 single stranded binding protein (SSB) **C.** The increased genomic alterations due to the genes' DNA methylation that alters their activity is shown. Additionally, *ASCC3* and *ALKBH3* work in opposite directions on the DNA strands. This figure entails the so far investigated behavior of DNA replication and damage reversal genes in GBM, although *ASCC3* and *ALKBH3* have yet to be investigated. This figure is hypothetical and based on prior research, therefore reflecting our hypothesis that *ASCC3* and *ALKBH3* abnormal methylation should lead to more genomic alterations. This figure was created in BioRender.com

study cohort consisting of a dataset of 254 patients. Only patients with mRNA expression and methylation data were considered. The parameters considered for this study were characterized into groups based on their mean values (**Table 1**). The continuous variables were found to be not normally distributed, discovered via the Shapiro-Wilk test.

In our final study cohort, we observed that most of the patients diagnosed with GBM are less than 60 years old, consisting dominantly of males (61.4%). Independent t-tests for age and sex showed no significance between any of the variables except for Karnofsky Patient Score (KPS), a scale that measures a cancer patient's ability to perform tasks in daily life (*P* = 0.0002). In independent t-tests, the variables methylation and mRNA expression of *ASCC3* and *ALKBH3*, FGA, and KPS were compared in the old versus young patients and male versus female patients to understand more about how demographics could affect the genetic factors.

Most patients (66.1%) had a lower FGA when the mean value for FGA of 0.2134 was considered. We observed 82.3% of patients had hypomethylation of *ASCC3*, while 61.8% of patients had a hypomethylation of *ALKBH3* (**Table 1**). The mean percentage of methylation (POM) was higher for *ALKBH3* (mean = 2.809%) when compared to *ASCC3* (mean = 1.842%). The mRNA expression of *ASCC3* and *ALKBH3* in most patients were low (67.9%; 82.2%), with more than 150 patients having mRNA expression fold change less than -1 for both genes (**Figure 2**).

Variables	Categories	Percentage
Age (n = 254)	<=60 y	54.7%
	>60 y	45.3%
Sex (n = 254)	Female	38.6%
	Male	61.4%
Karnofsky Patient Score (KPS)	<=74	34.9%
T-test *p*-value (Age vs KPS) = 0.0002 T-test *p*-value (Sex vs KPS) = 0.9447	>74	65.1%
Fraction Genome Altered (n = 254) (FGA)	<=0.2134	66.1%
T-test *p*-value (Age vs FGA) = 0.8585 T-test *p*-value (Sex vs FGA) = 0.8684	>0.2134	33.9%
ASCC3 methylation (n=254) [percentage of methylation]	<=1.842%	82.3%
T-test *p*-value (Age vs *ASCC3* methylation) = 0.4483 T-test *p*-value (Sex vs *ASCC3* methylation) = 0.2605	>1.842%	17.7%
ASCC3 mRNA (n = 253) [Z scores]	<= -0.7417	67.9%
T-test *p*-value (Age vs *ASCC3* mRNA) = 0.7553 T-test *p*-value (Sex vs *ASCC3* mRNA) = 0.427 Mean expression: -0.7352	> -0.7417	32.1%
ALKBH3 methylation (n = 254) [percentage of methylation]	<= 2.809%	61.8%
T-test *p*-value (Age vs *ALKBH3* methylation) = 0.3702 T-test *p*-value (Sex vs *ALKBH3* methylation) = 0.4594	> 2.809%	38.2%
ALKBH3 mRNA (n = 253) [Z scores]	<= -1.002	82.2%
T-test *p*-value (Age vs *ALKBH3* mRNA) = 0.6405 T-test *p*-value (Sex vs *ALKBH3* mRNA) = 0.493 Mean expression: -1.002	> -1.002	17.8%

Table 1: Cohort characteristics of patients in the dataset. All variables studied were split into two categories based on mean values, except for sex, which was split into male and female. The percentage of patients that fall into one of the two categories is shown on the right column. The total samples analyzed are shown next to the variable name, and only significant t-test values are displayed.

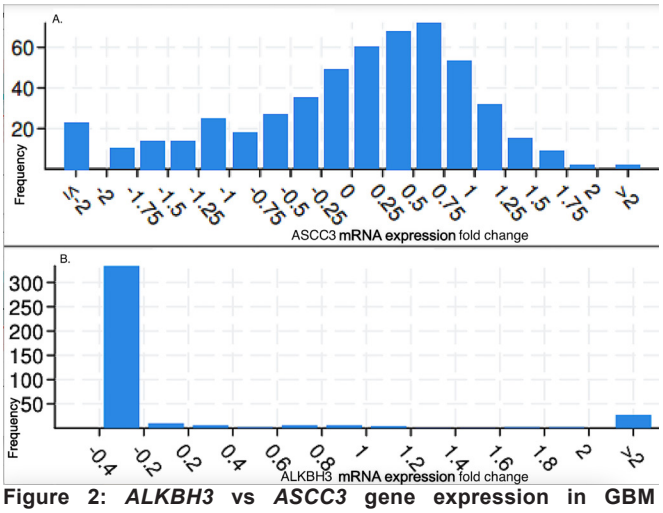

Figure 2: *ALKBH3* vs *ASCC3* gene expression in GBM categorized into high, low, and mean expression. The **A.** *ASCC3* and **B.** *ALKBH3* mRNA expression fold change of all patients in the dataset is represented by the histogram. 254 patient samples are represented. The x-axis represents the expression for the two genes while the y-axis represents the frequency of patients with the specific range of mRNA expression. Low fold change is below -1, while -1 to 2 displays middle or average fold change, and greater than 2 shows the high mRNA expression samples.

Correlation of *ASCC3* and *ALKBH3* gene with FGA

The relationship between *ASCC3* methylation, mRNA expression fold change, and FGA was analyzed (**Figure 3**). There is an inverse correlation between the following: [i] a significant correlation of *ASCC3* methylation and *ASCC3* mRNA expression (ρ = -0.17, *P* = 0.019; **Figure 3A**); [ii] an insignificant correlation of *ASCC3* methylation and FGA (ρ = -0.063, *P* = 0.38; **Figure 3B**); [iii] *ASCC3* mRNA expression and FGA (ρ = -0.028, *P*= 0.7; **Figure 3C**).

The relationship between *ALKBH3* mRNA expression and DNA methylation demonstrated an opposite effect on FGA (**Figure 3F**). The Firehose Legacy study, whose dataset we used for our study, measured FGA using the following parameters: the number of DNA sequence changes, copy number aberrations, and chromosomal rearrangements. There were positive correlations between the following: [i]. a non-significant correlation between *ALKBH3* methylation and *ALKBH3* mRNA expression (ρ = 0.06, *P* = 0.4; **Figure 3D**); [ii]. *ALKBH3* methylation and FGA (ρ = 0.03, *P* = 0.67; **Figure 3E**); [iii]. *ALKBH3* mRNA expression and FGA (ρ = 0.13, *P* = 0.068; **Figure 3F**). However, due to *P*-values greater than 0.05 (**Figure 3B, 3C, 3D, 3F**) and only two p-values approximating 0.05 (**Figure 3A, E**), we cannot assume this data has a prominent correlation.

ASCC3 and ALKBH3's Impact on Disease-Free Status (DFS)

Hypomethylation of *ASCC3* was seen to be a significant indicator for slower disease progression (*P* = 0.039), indicating that there is a potential prognostic role (**Figure 4A; Table 2**). Additionally, *ASCC3* methylation had a median DFS (hypermethylation-median=13.6 months; hypomethylation-median=26.1 months) ratio of 1:1.9 between hypermethylation and hypomethylation. However, *ASCC3* mRNA expression, along with both *ALKBH3* methylation and mRNA expression

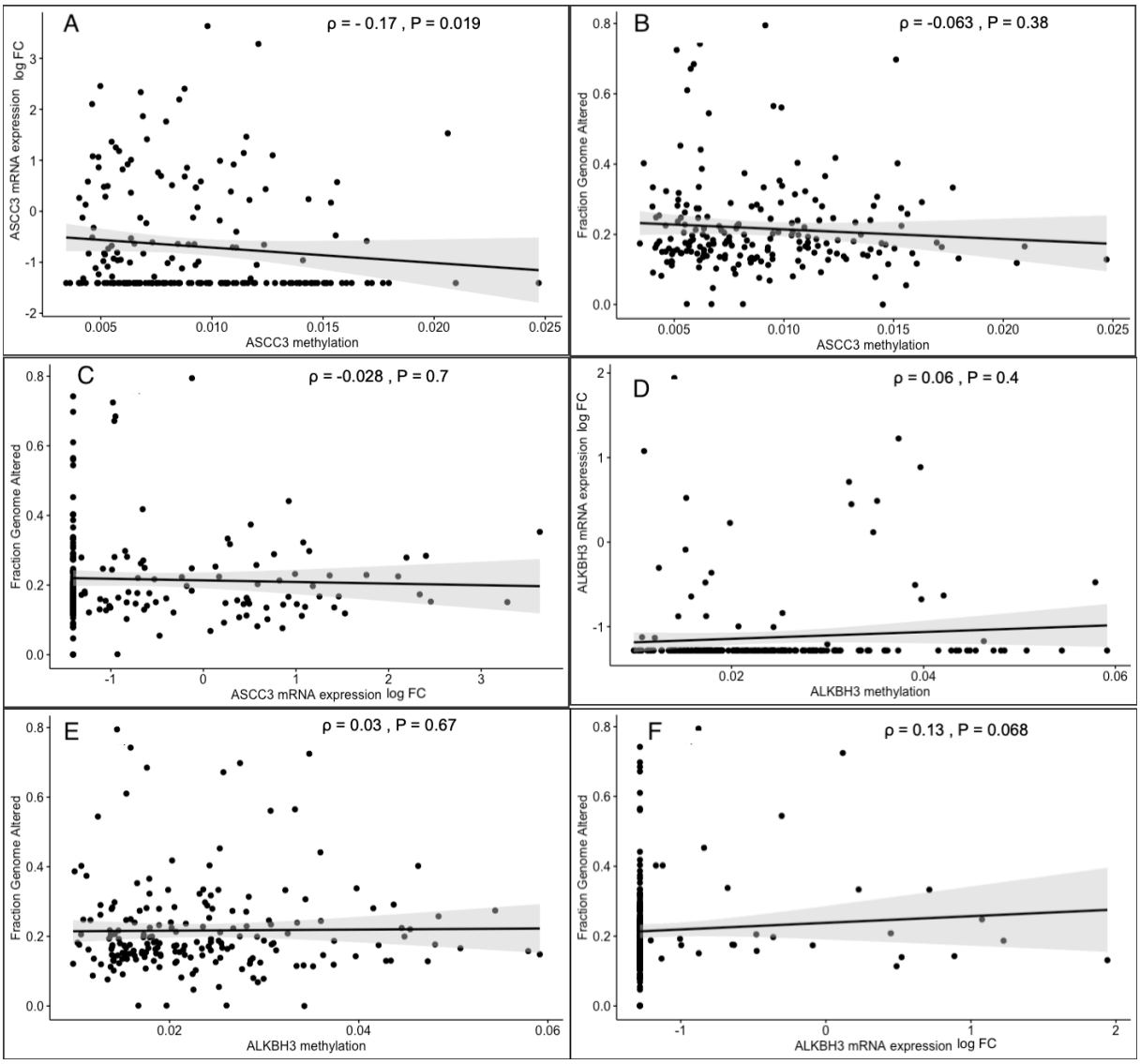

Figure 3: *ASCC3* and *ALKBH3* DNA methylation vs mRNA expression, DNA methylation vs FGA, and mRNA expression vs FGA. Data is in fraction of promoter region methylated for DNA methylation and in transcripts per million (TPM) for mRNA expression. A log2 transformation was performed on DNA methylation data. rho-values and *P*-values are shown at the top of each figure. The value of 254 data points are represented. Each subfigure showcases linear regressions for one of the following: **A.** *ASCC3* methylation and *ASCC3* mRNA expression, **B.** *ASCC3* methylation and FGA, **C.** *ASCC3* mRNA expression and FGA, **D.** *ALKBH3* methylation and mRNA expression, **E.** *ALKBH3* methylation and FGA, and **F.** *ALKBH3* mRNA expression fold change and FGA.

did not indicate any significant prognostic impact for DFS (*P* > 0.05) (**Figure 4B–D**), the ratios for median months between hyper:hypo / high:low variables are close to 1:1 (**Table 2**).

ASCC3 and ALKBH3's Impact on Overall Survival (OS)

ASCC3 methylation along with the *ALKBH3* methylation and mRNA expression did not indicate any significance for OS (*P* > 0.05), for the median months between hyper: hypo / high: low variables are close to 1:1 (**Figure 5A, C, D**) (**Table 3**). However, A higher mRNA expression of *ASCC3* mRNA expression was seen to be a significant indicator for better survival (*P* = 0.05) **(Figure 5B; Table 3**). Additionally, *ASCC3* mRNA expression had a median overall survival time (high expression-median=22.8 months; low expression-median= 43.4 months) that has a ratio of 1:1.9 between the high and low groups.

DISCUSSION

GBM is known to be the most lethal and aggressive brain malignancy (1). Despite being rare, it has been a topic of study for many years (1). However, due to its complexity, very little is understood about GBM and only one strong biomarker, *MGMT* methylation status, has been identified in these years of research (1). It is therefore essential to identify more genes that have a potential role in developing GBM (1). We therefore selected genes with a similar function to *MGMT* and explored their respective potential as biomarkers (10). We aimed to determine whether methylation and mRNA expression of *ASCC3* and *ALKBH3* have a significant biological and prognostic role in GBM. *ASCC3* and *ALKBH3* are both involved in repairing alkylation damage, a harmful chemical alteration to DNA that often causes cell death, making them similar in

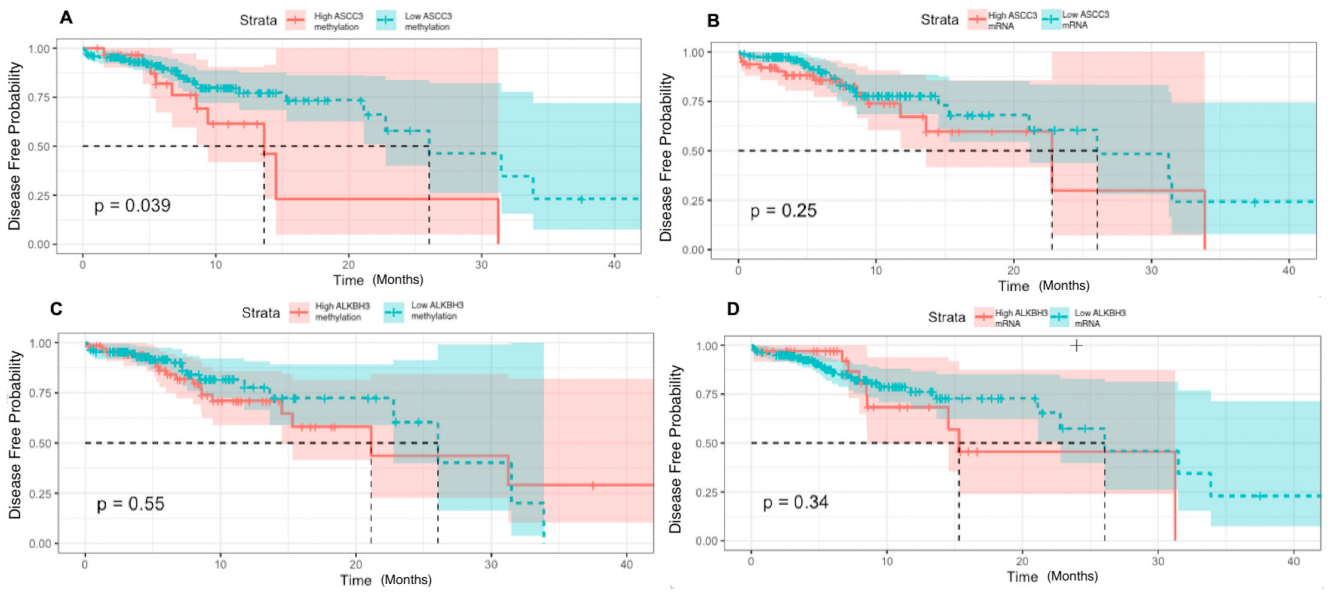

Figure 4: Relation of *ASCC3* and *ALKBH3* DNA methylation and mRNA expression with DFS. A. Hyper and hypo *ASCC3* methylation's relation to the statistical probability. **B.** High and Low *ASCC3* mRNA expression's relation to the statistical probability. **C.** Hyper and hypo *ALKBH3* methylation's relation to the statistical probability. **D.** High and Low *ALKBH3* mRNA expression's relation to the statistical probability. Data is represented in the probability of being disease free over a period of months. *P*-values are shown in the figures. Grey dotted lines represent the median number of months while remaining disease free for hyper/hypo methylation and low/high mRNA expression.

function to *MGMT* (12). To the best of our knowledge, this is first time the methylation status and the corresponding fold change values for these two genes have been tested in GBM using *in silico* analysis, though they have been studied in other conditions. *ALKBH3* has been found to be overexpressed in salivary gland carcinoma and prostate cancer. The expression profile of *ASCC3* studied across different tissues has revealed that *ASCC3* does not have any significant expression in cancers (17, 18).

We began our analysis to understand the cohort characteristics, and we found that the KPS has significant changes with age, as per previous studies and common knowledge entail (19). It is known that differences in age contribute to a patient's ability to respond to cancer (20). Cancer develops more slowly in older patients, but the patient's ability to defend against cancer decreases, and DNA repair pathway's ability (21, 22). This study shows that men have a 1.57 times greater chance of being diagnosed with GBM, compared to women in accordance with previous studies (4).

After understanding the patient characteristics, we studied the methylation and expression of *ALKBH3* and *ASCC3* genes. Our analysis revealed that more patients have hypomethylation of *ASCC3* and *ALKBH3*, and there are more patients who have low mRNA expression. These observations may likely suggest the low mRNA expression of these genes, despite there being hypomethylation. The observation indicates a dysfunctional molecular mechanism which may be a cause for the faulty alkylation DNA repair. This dysfunction could potentially be a factor for gliomagenesis. The sample size of the dataset could have potentially played a role in producing insignificant *P*-values for the comparison t-tests. Potential modifications to improve the results of this study are conducting ANOVA tests with more divisions of age groups and investigating the *MGMT* gene to reference to past studies. However, the division of

Variable (n)	Events (disease progression)	Median (months)	*P*-value
ASCC3 hypermethylation (30)	25	13.6	0.039
ASCC3 hypomethylation (152)	38	26.1	
High *ASCC3* mRNA expression (64)	15	22.8	0.25
Low *ASCC3* mRNA expression (117)	22	26.1	
ALKBH3 hypermethylation (70)	18	21.1	0.55
ALKBH3 hypomethylation (112)	19	26.1	
High *ALKBH3* mRNA expression (34)	9	15.3	0.34
Low *ALKBH3* mRNA expression (147)	28	26.1	

Table 2: Kaplan Meier Test to find probability of being disease-free for groupings of *ASCC3*, *ALKBH3* methylation and mRNA expression. Data is represented in the probability of being disease free over a period of 44 or less months. Although the highest number of disease-free months for a patient was 43.2, the values of disease-free months varied for all patients, with some patients having as low as 0.1 disease free months. The effect of the variables in the left column on a patient's disease-free Status over a period of months is shown. The events column represents the number of patients who were disease free at certain times. The reported median value represents the median number of disease-free months in the patient cohort. *P*-values were calculated through the Kaplan Meier test based on the difference between the statistical probability for the tested groups. (n) is the total number of data points.

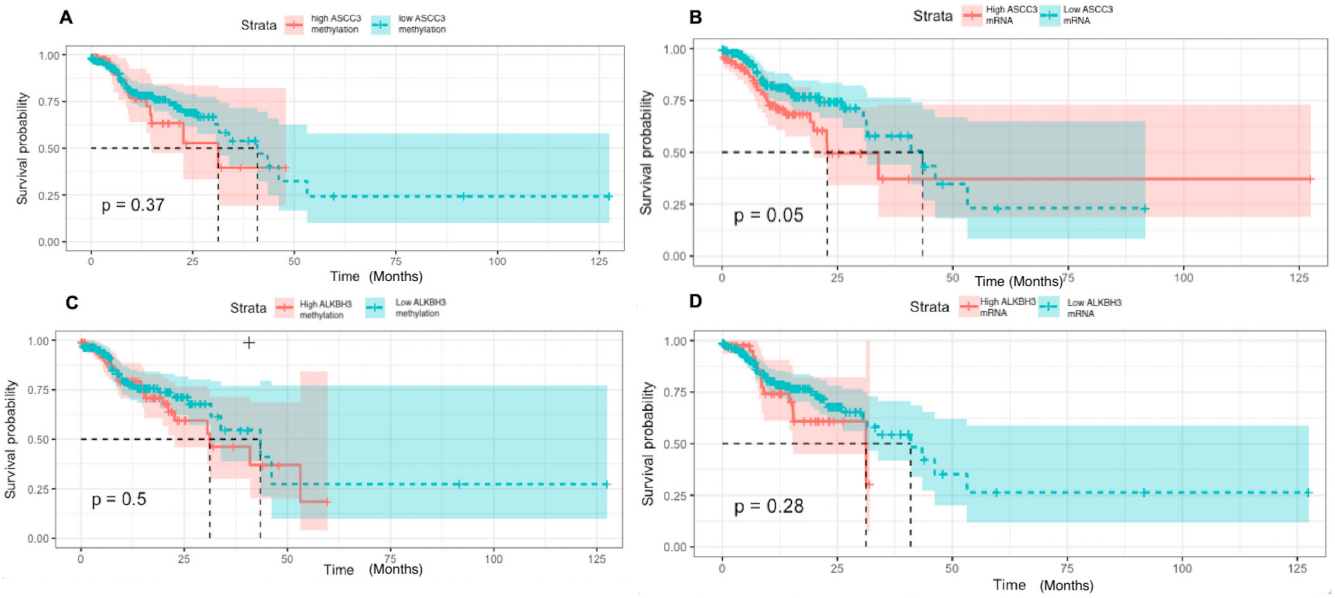

Figure 5: Relation of *ASCC3* and *ALKBH3* DNA methylation and mRNA expression with Overall Survival. Hyper and hypo *ASCC3* methylation's relation to the probability (**A**), high and Low *ASCC3* mRNA expression's relation to the probability (**B**), hyper and hypo *ALKBH3* methylation's relation to the probability (**C**), high and Low *ALKBH3* mRNA expression's relation to the probability (**D**). Data is represented in the probability of survival during months after diagnosis. *P*-values are shown in the figures. Grey dotted lines represent the median number of months alive.

patients does not shed light on the role and the interplay of these genes, which is explained further below.

Researchers have shown that mRNA expression and DNA methylation are inversely correlated (23, 24). To delve further into how DNA methylation of these genes is a regulator in reference to mRNA expression, correlation tests were conducted between *ASCC3*, *ALKBH3* methylation and mRNA expression. Notable findings include a negative correlation between *ASCC3* methylation and mRNA expression, which despite having a *P*-value slightly above 0.05, shows potential inverse patterns. The findings could imply that *ASCC3* functions as biologically expected in the context of this analysis; however, there may be other factors like transcriptional and post-transcriptional modifications that could affirm or negate these results. However, studying these factors was not within the scope of this study. Regardless, the negative correlation between *ASCC3* methylation and mRNA expression of *ASCC3* may also suggest that a lower *ASCC3* methylation paired with a higher *ASCC3* mRNA expression could allow for increased DNA repair in GBM, therefore decreasing FGA. Contrastingly, the correlation discovered between *ALKBH3* methylation and mRNA expression is positive albeit supportive of the null hypothesis. The correlation may indicate potential errors in the interplay between epigenetics and genetics in *ALKBH3* in GBM, as *ALKBH3* demonstrated a weakly significant positive correlation between DNA methylation and mRNA expression. As mentioned for *ASCC3* above, there could be other transcriptional factors that may impact the overall functionality of *ALKBH3.* Interestingly, this pilot study points to the contrasting functionality of these two genes in GBM. The contrast may potentially suggest the underlying dysfunction of alkylation damage reversal pathway in GBM. The biological functionality of *ASCC3* in GBM paired with the unexpected direct relationship between mRNA expression and

Variable (n)	Events (death)	Median (months)	*P*-value
ASCC3 hypermethylation (45)	13	31.2	0.37
ASCC3 hypomethylation (207)	50	40.9	
High *ASCC3* mRNA expression (81)	25	22.8	0.05
Low *ASCC3* mRNA expression (170)	38	43.4	
ALKBH3 hypermethylation (97)	28	31.2	0.5
ALKBH3 hypomethylation (155)	35	43.4	
High *ALKBH3* mRNA expression (45)	13	31.2	0.28
Low *ALKBH3* mRNA expression (206)	50	40.9	

Table 3: Kaplan Meier Test to find probability of overall survival for groupings of *ASCC3*, *ALKBH3* methylation and mRNA expression. Data is represented in the probability of survival during months after diagnosis, with the highest number of months of survival for a patient being 127.5 and the lowest months of survival being 0.1 months. The effect of the variables in the left column on a patient's overall survival status over a period of months is shown. The events column represents the number of patients who survived at certain times. The reported median value represents the median number of months the patients in the patient cohort. *P*-values were calculated through the Kaplan Meier test based on the difference between the statistical probability for the tested groups. (n) is the total number of data points.

methylation for *ALKBH3* suggests that collaboration between the two genes in GBM could be flawed. Further research like conducting *in vitro* studies in the future using primary glioma cells or malignant GBM cell lines to determine the mechanism of action for these two genes, *ASCC3* and *ALKBH3,* could contribute to better knowledge on their functions.

To understand the observed contrasting correlations and study the potential effect of this DNA repair dysfunction on genomic alterations further, we performed linear correlation tests between the DNA methylation and mRNA expression of *ASCC3* and *ALKBH3*, respectively, with FGA. The data showed similar trends to the previous analysis, as both *ASCC3* methylation and mRNA expression were inversely correlated with FGA. The correlation of *ASCC3* methylation with FGA was expected to be positive, due to the inverse correlation found in the previous analysis; however, the opposite was observed. The key finding from these correlation tests were that patients with high *ASCC3* mRNA expression demonstrate a high FGA and *vice versa*. The correlation tests may indicate that high *ASCC3* is potentially not beneficial for genome stability.

On the other hand, both *ALKBH3* methylation and mRNA expression are positively or directly correlated with FGA. The positive correlation possibly demonstrates that higher mRNA expression of *ALKBH3* increases the genomic alterations, indicating a divergence from *ALKBH3*'s biological role of decreasing genetic damage. Contrastingly, *ALKBH3's* positive correlation with FGA could also be a potential attempt to combat the increased DNA damage in GBM by upregulating *ALKBH3.* Most of the results here demonstrate contradicting findings, as correlation tests merely demonstrate a trend as opposed to conclusive evidence. It is very important to notice that our findings clearly illustrate the epigenetic and genetic dysregulation of the DNA damage reversal repair mechanism in Glioblastoma. *ASCC3's* potential role in gliomagenesis prevention and *ALKBH3*'s potential role in attempting to correct the damage caused by the disease suggest that these are key findings. The findings could validate that there are multiple underlying DNA damages in GBM in addition to abnormal *MGMT* methylation.

Our final analysis was to test whether *ASCC3* and *ALKBH3* had the ability to predict DFS and OS, like *MGMT* methylation status' abilities as a significant and strong biomarker. Our results demonstrate that *ASCC3* hypomethylation along with a higher mRNA expression result in a significantly better DFS and improved OS, respectively. The significance of hypomethylation for *ASCC3* possibly indicates that the DNA repair by *ASCC3,* when functional, may lead to better patient recovery. However, this theory needs to be tested further to see if *ASCC3* has a role in therapeutic response. Additional tests could include a comparison of *ALKBH3* and *ASCC3's* behavior in glioblastoma with *MGMT or* analyzing *ALKBH3* and *ASCC3* in lower grade gliomas to understand their changes in activity and roles in carcinogenesis. *ALKBH3*, however, did not show any such significance in our analysis, potentially indicated in our earlier results about *ALKBH3* being more dysfunctional among the two genes.

In summary, this study has shown the need to investigate online datasets for relatively under-researched genes that may aid understanding the complexity of GBM. These genes have the potential to demonstrate our study's discovered trends of the dysfunction of *ASCC3* and *ALKBH3* and the significance of the *ASCC3* gene in DFS and OS. However, the discovery also emphasizes the need to research and validate these findings using *in vitro* and *in vivo* experimental models in the future, which will enable a better understanding of *ASCC3* and *ALKBH3*'s roles in GBM, as well as their potential as future biomarkers.

MATERIALS AND METHODS

Data download

This *in silico* study was conducted using publicly available cBioportal and TCGA database, licensed by the National Institute of Health (NIH), USA. Glioblastoma Multiforme experimental data set (TCGA, Firehose Legacy, 2012) with 619 samples was chosen. The data files used were 'data_methylation_hm450', 'data_methylation_hm27', 'data_expression_all_sample_Zscores', and 'data_bcr_clinical_data_patient'.

Data processing

The dataset was sorted and filtered to specifically test the hypothesis by applying our inclusion and exclusion criteria. The inclusion criteria were used to obtain fraction of genome data from the overall clinical data only for patients who had data for the following, methylation_hm450, and mRNA expression Z-scores. The inclusion criteria was used to include all the patients with data for all of the following variables: Patient ID, age, sex, FGA, mutation count, DFS, OS, Karnofsky Patient Score (KPS), and *ASCC3* and *ALKBH3* mRNA expression and methylation. *ASCC3* and *ALKBH3* methylation and mRNA expression data were obtained from the 'data_methylation_hm27' and 'data_methylation_hm450' files and moved to data_bcr_clinical_data_patient excel spreadsheet. All the sorted and filtered data was compiled into a working dataset in Excel to import into R software.

Data analysis

The statistical analysis was conducted using RStudio (Version 1.3.1056). The data was first characterized via frequency distribution after finding the mean and median values for each variable from the data. All variables were grouped based on their mean. The percentages of these groups out of the total data points for the variable were found. An independent sample t-test was conducted between patient age and sex vs FGA, mutation count, mRNA expression, and methylation in a manner such that the continuous variables were compared for patients who were either old vs. young or male vs. female (**Table 1**). Shapiro-Wilk normality test was conducted to check the distribution of the data for the variables FGA, Mutation Count, *ASCC3* and *ALKBH3* mRNA methylation and expression. One-way ANOVA test was conducted for 5 age categories of 18 to 30 years, 31 to 45 years, 46 to 60 years, 61 to 75 years, and 76 to 90 years. The test compared the *ASCC3* and *ALKBH3* methylation, mRNA and KPS for the 5 age categories. Box plots were generated to visually represent the ANOVA and the t-test. A histogram was created for a visual representation of the *ASCC3* and *ALKBH3* mRNA expression using cBioportal.

We converted the methylation values into POM, additionally we also categorized the samples for methylation as hyper/hypo methylated. Similarly, we divided the samples into high and low mRNA expression, to test for the association between methylation, mRNA expression and FGA. The Spearman's rank correlation test was used to analyze the correlation

between FGA and mutation count with *ASCC3* and *ALKBH3* mRNA methylation and mRNA expression. Significance was determined by identifying p-values less than 0.05. Scatterplots were made upon conducting the correlation test for a graphical representation. The Chi-squared test was conducted for low vs high levels of FGA with the low and high levels of the gene variables (methylation and mRNA expression). The Kaplan Meier Survival Test was conducted using the overall survival months (OSM) and overall survival (OS) with the high and low groups of Sex, Age, *ASCC3* and *ALKBH3* mRNA, and KPS.

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