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Article

Mismatch repair is not correlated with genomic alterations in glioblastoma patients

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SUMMARY

Glioma is the most common brain tumor of the glial cells with a highly infiltrative nature. A grade IV glioblastoma is the most malignant and aggressive presenting with complex mutations and a very high genomic alteration status. Glioblastoma patients have the fastest progression compared to other cancer patients and poor survival. Methylation status of *MGMT*, a gene encoding a DNA repair enzyme, is the only known promising biomarker. There is a need to look for new biomarkers in similar functional pathways. Two mismatch repair pathway (MMR) genes, *hMSH2*, and *hMSH6*, which function downstream of *MGMT,* appear as ideal targets. We hypothesized that a lower expression of *hMSH2* and *hMSH6* are associated with a higher fraction of genome-altered (FGA) which would influence survival. We conducted an *in silico* study using publicly available datasets consisting of clinical and experimental data. We obtained a weak positive correlation between *hMSH2* and *hMSH6* with FGA. Sex specific analyses yielded a stronger significant positive correlation. However, overall the measures of correlation were too small as the component analysis indicated homology and validated the findings. Additionally, the expression of *hMSH2* and *hMSH6* did not affect overall survival. We conclude that in this cohort *hMSH2* and *hMSH6* did not indicate biological significance in causing FGA, nor were they effective biomarkers for survival as hypothesized.

INTRODUCTION

Glioma is the most complex type of brain tumor in the brain's glial cells. The glial cells, which include astrocytes, oligodendrocytes, and microglia, support the nervous system and outnumber the neurons by three times (1). Glioma occurs in four distinct grades (I, II, III, and IV), classified based on their potency; grades III and IV are considered malignant glioma and are highly aggressive. Grade IV, known as glioblastoma, is the most malignant and accounts for about 54% of all brain tumors, making it the most common (2). Glioblastoma is extremely hard to treat due to its highly infiltrative nature—it does not have clear borders, making it difficult to excise the complete tumor surgically (3). Additionally, glioblastomas can occur *de novo*, meaning they can appear in grade IV without progressing through the other stages (2).

The above factors make glioblastoma extremely deadly, and cases are almost always lethal, with patients surviving on average less than 15 months after treatment (2). Fewer than 5% of people survive 5+ years after being diagnosed (2). Treatment for glioblastoma primarily consists of surgery followed by concurrent radiotherapy and chemotherapy with Temozolomide (4). However, it is not entirely adequate; reports indicate that the efficacy of this treatment module works better when *MGMT,* the only direct reversal DNA repair gene, is hypermethylated, which is seen in about 40–60% of patients (5). Therefore, there is an emergent need to identify new markers which could be used for understanding glioblastoma better and developing newer forms of therapy.

One of the primary causes of cancers, including glioblastoma, are mutations that occur during the cell replication cycle (6). One small mutation can be passed on through this cycle multiple times until there are many cells with this mutation (7). Among the significant mutations that occur are insertion-deletion errors and base-base mismatches. These mutations cause genomic alterations. Insertion errors occur when extra base pairs are inserted, while deletion errors occur when base pairs are deleted mistakenly (6). Base-base mismatches occur when bases are improperly matched to each other. The mismatch repair pathway (MMR) is responsible for detecting and repairing these errors (8). A dysfunctional MMR potentially leads to mismatch errors and genomic alterations, which means the fraction of the genome altered (FGA) is greater. FGA is a measure that indicates the extent of genomic alteration and severity of cancer (9). Glioblastoma, being the most complex genomically among all tumors potentially harbors the highest genomic alterations (10). The MMR pathway acts downstream of MGMT, which is encoded by the *MGMT* gene, the methylation status of which is the most prominent prognostic and survival biomarker in glioblastoma (11,12). It would be worth evaluating if MMR may have a similar effective role as a biomarker. In addition, studying alternate DNA repair mechanisms is essential, as it may also play a role in therapy response to alkylating agents, the current standard of treatment for glioblastomas.

Two essential genes of MMR are human Mut S homolog 2 (*hMSH2*) and Mut S homolog 6 (*hMSH6*). These genes code for proteins that interact to form a heterodimer called MutSα (13). Together they are responsible for the detection of insertion-deletion errors and base-base mismatches. Both the *hMSH2* and *hMSH6* are essential in MMR and mismatch recognition will be impacted without their effective function (14). Previously mutations in *hMSH2* and *hMSH6* have been correlated with prostate cancer, endometrial carcinoma, and colorectal cancer, to name a few (15,16). In this *in silico* study (studies performed virtually using publicly available datasets),

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we determined the effect of the MMR pathway on glioblastoma by analyzing the correlation of mRNA and corresponding protein expression of two critical MMR genes, *hMSH2* and *hMSH6*, with the fraction of genome altered (FGA) using the patient data obtained from the datasets. We analyzed the genes' roles as a potentially good indicator of functional outcomes (or biomarkers) for glioblastoma by analyzing their predictive efficacy. We hypothesized that a lower expression of *hMSH2* and *hMSH6* is associated with a higher fraction of FGA influencing poor survival.

From the analysis performed, we determined a non-significant correlation between *hMSH2* and *hMSH6* gene expression with FGA. Therefore, our hypothesis is rejected and shows that these genes appear to affect the FGA to a much lesser extent than we expected. Our data indicate that both genes cannot accurately predict prognosis or survival, so they are not good indicators of functional outcomes for glioblastoma.

RESULTS

Cohort Characteristics and expression values

The mean age for the chosen cohort of 366 glioblastoma patients was 59 years, and 61.7% were males. Our cohort characteristics were very similar to other research concerning the mean age and males having a higher incidence of the disease (17). The Karnofsky Performance Status (KPS) is a measure of the functional statuses of patient. A score of 80% or higher for a patient is considered good, and indicates that they are able to live normally with some to no difficulty (18). The KPS is good for 71.73% of the cohort, and their mean age is 55 years lower than the overall cohort based on the rationale established before (19). We found that the overall survival was relatively low, with 83.29% not surviving for five years after diagnosis (**Table 1**). Expression distribution of the two MMR genes for their mRNA and protein expression values are also reported (**Figure 1**). The mean and standard deviation values are as follows. Gene: *hMSH2* (0.029 ± 1.024), *hMSH6* (0.030 ± 1.027); protein: MSH2 (0.055 ± 0.888) and MSH6 (0.037 ± 0.863). Some variables reported in **Table 1** do not have data for all patients in the cohort.

Variables (n)	Relative Frequency (%)	Mean Age
Age (n=366)		58.88
≤58 years	45.36	
>58 years	54.64	
Sex (n=366)		
Male	61.74	59.30
Female	38.25	58.20
Karnofsky Performance Score (n=276)		
0-40% (Poor)	3.62	66.60
50-70% (Moderate)	24.27	64.35
80-100% (Good)	71.73	55.84
Overall Survival Status 5 years after diagnosis (n=365)		
Dead	83.29	58.65
Alive	16.71	56.32

Table 1. Cohort Clinical Characteristics (in %). Each variable is split into categories, and the relative frequency of each group was calculated. The mean age of each group is shown on the right. The total number of patients is given for variables with fewer data points. For KPS, 0-40%: (Death to Disabled); 50-70%: (Frequent Medical Care to Caring for Self); 80-100% (Normal Activity with Some Difficulty to Normal Health)

Correlation and clustering analysis

To validate the linearity between mRNA expression & respective protein expression with FGA, we performed a Spearman correlation test with 0.05 as the p-value threshold. We observed a positive correlation ρ (correlation coefficient) between *hMSH2* and MSH2 expression *(*ρ=0.429; p=5.36 x 10^{-7}). Similarly, we found *hMSH6* to have a significant positive correlation with MSH6 (ρ= 0.47; p-value: 5.64 x 10^{-8}). This suggests that the transcriptional and translational control of gene expression is optimally regulated and coupled.

Our next goal was to see if *hMSH2/6* play a critical role in altering the genome through correlation analysis. We found a very low (magnitude) positive correlation of 0.08 between *hMSH2* mRNA expression and FGA (p-value: 9.59 x 10^{-2}) (**Figure 2a)**. MSH2 protein expression had a much higher positive correlation that was statistically significant (ρ = 0.31; p-value: 2.7 x 10^{-4}) for correlation with FGA (**Figure 2g**). Interestingly, we observed *hMSH6* mRNA to be slightly more positively correlated with FGA than *hMSH2* (ρ = 0.17; p-value: 5 x 10^{-4}) (**Figure 2b**). For MSH6 protein (**Figure 2g**), the correlation is higher than the correlation for the gene (ρ=0.32; p-value= 2.5 x 10^{-4}). Although there were a few statistically significant results obtained, overall the magnitude of correlation was not strong (small correlation coefficients) as demonstrated in **Figure 3**.

We further analyzed the correlations between the sexes to better understand the previous result and found that in females the correlation was positive and poor for both *hMSH2* and *hMSH6* (ρ = 0.06 & 0.02; and p-value = 4.5 x 10^{-1} & 7.7 x 10^{-1}, respectively) (**Figure 2c, d**). However, males demonstrated a non-significant negative correlation for *hMSH2* mRNA with FGA (ρ = -0.05, p-value = 4.4 x 10^{-1}) (**Figure 2e**).

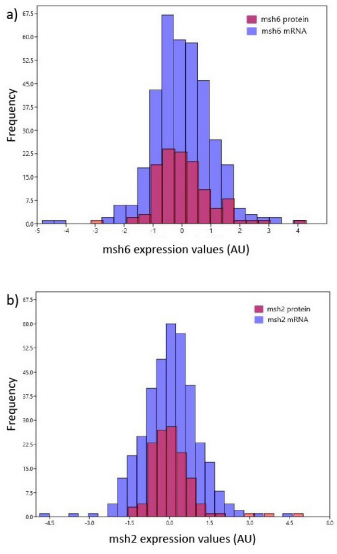

Figure 1. Histogram plots for gene and protein expression. Plots for distribution of mRNA/protein expression values for **a)** hMSH6 and **b)** hMSH2 following a gaussian trend. The magenta bar represents protein expression distribution in samples, while the purple represents mRNA expression.

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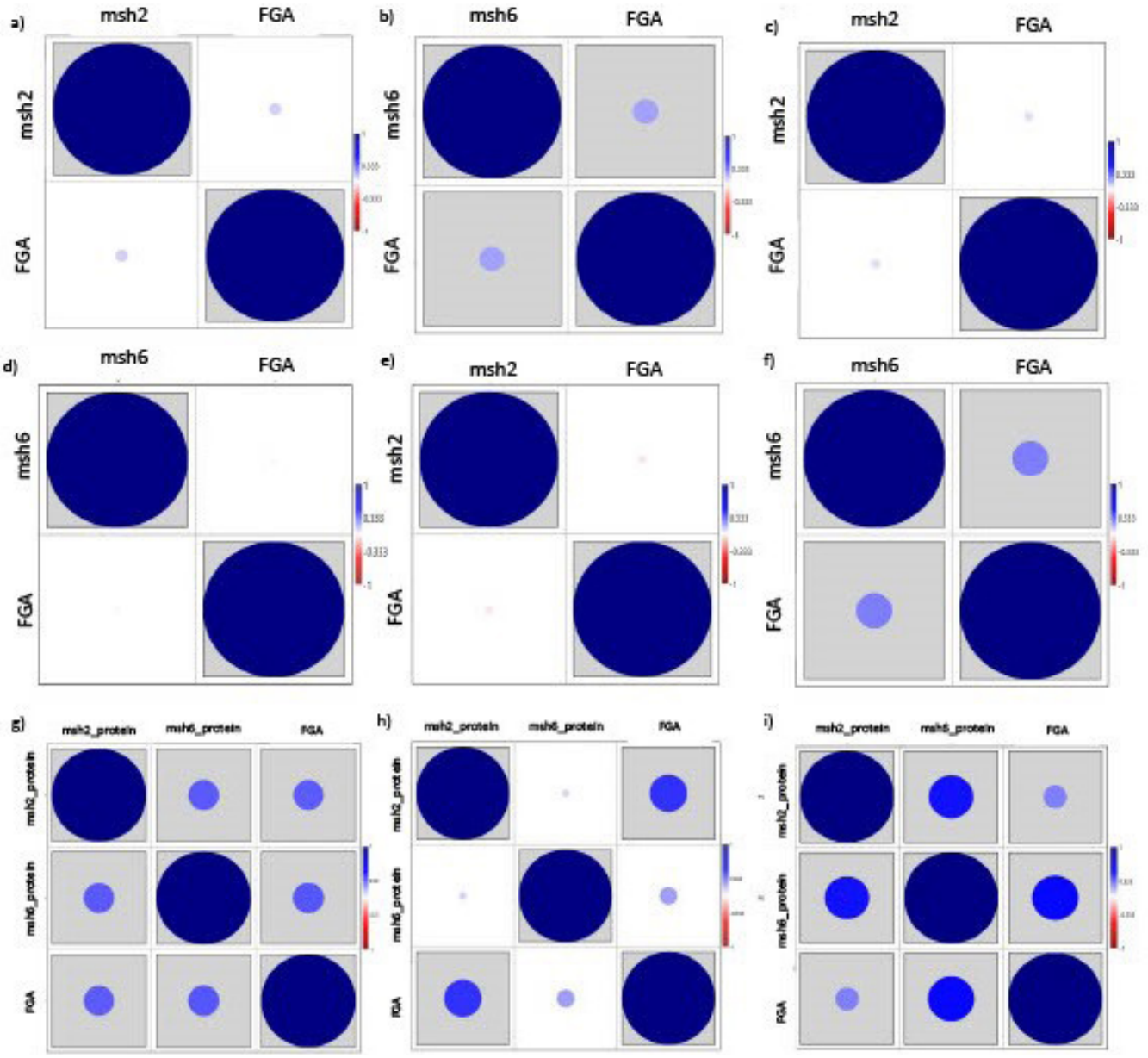

Figure 2. Correlation plots of hMSH2 and hMSH6 mRNA with FGA and MSH2/6 protein levels with FGA. Plots depict a biologically not significant correlation of MSH genes expression with FGA. **T**he blue circles represent the correlation intensity (blue is the strong positive correlation and vice-versa for red). The grey boxed circles represent a statistically significant correlation; also, bigger the diameter, better the correlation magnitude. **a-b)** The overall hMSH2: (ρ=0.08; p-value: 0.0959), hMSH6: (ρ = 0.17; p-value: 0.0005). **c, d)** Correlation of female patients' gene expression with FGA hMSH2 and hMSH6 (ρ = 0.06 & 0.02, respectively). **e, f)** Correlation of male patients' gene expression with FGA, hMSH2 and hMSH6 (ρ = -0.05 & 0.025215, respectively). Similarly, the females and males MSH2 & 6 protein expression correlation with FGA for **(g)** overall, **(h)** females and **(i)** males.

Interestingly, *hMSH6* mRNA had a statistically significant positive correlation with FGA (ρ = 0.25215; p-value: 1.0 x 10^{-4}) (**Figure 2f**). The analysis for protein with FGA largely revealed statistically significant positive correlation (MSH2 males: ρ = 0.24, p-value: 0.03; females: ρ = 0.4, p-value: 4 x 10^{-3}) (**Figure 2h, i**). MSH6 protein showed significance and higher positive correlation for males (ρ = 0.48; p-value: 1 x 10^{-5}) as seen in (**Figure 2i**), whereas females showed a lower non-significant correlation (ρ = 0.18; p-value: 2 x 10^{-1}) for MSH6 protein (**Figure 2h**). The difference in correlation values among sexes indicate the diversity and complexity of the tumors.

To understand our data further, we next performed a Principal Component Analysis (PCA) analysis on five variables of interest: age, sex, *hMSH2* gene expression, *hMSH6* gene expression, and FGA to determine overall data clusters in the dataset (**Figure 4**). We were interested in obtaining more meaningful observations among smaller relevant groups. PCA analysis showed no significant clustering of the data points outside the origin, indicating a single dominant trend across the samples pool, therefore suggesting the need to rule out further investigations.

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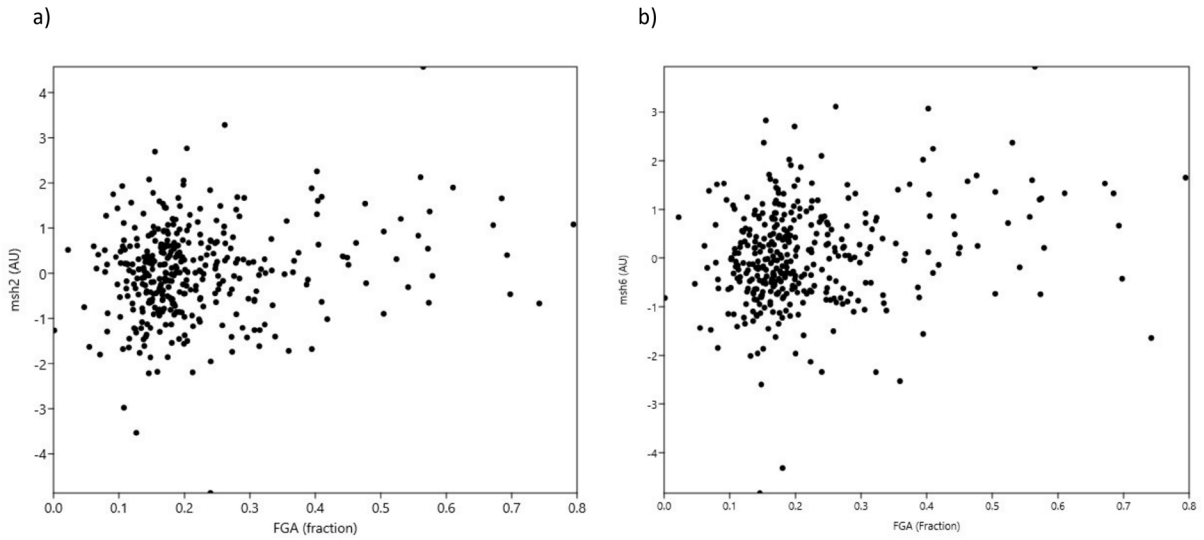

Figure 3. Expression distribution plot. There is no apparent correlation between **(a)** *hMSH2* **or (b)** *hMSH6* expression and FGA axes.

Survival Analysis - Overall Survival Status (OSS)

We performed Kaplan-Meier tests to determine the role of both mRNA and their respective protein expression of these two MMR players on patient survival. The Kaplan-Meier curve is a graphical representation that generally estimates the survival function from censored data, truncated, or missing values. It indicates the probability of a subject surviving upto a time. The analysis indicated no significant impact of mRNA expression of both these genes on overall survival. Similarly, MSH6 protein expression does not have an impact on survival (**Figure 5)**. However, it is of interest that higher MSH2 protein expression does show an improved survival (median=15.64 months) when compared to low MSH2 expression (median=13.27 months) although statistical significance (p=0.056) was not achieved. This finding offers exciting potential to explore further in a future study with a larger sample size.

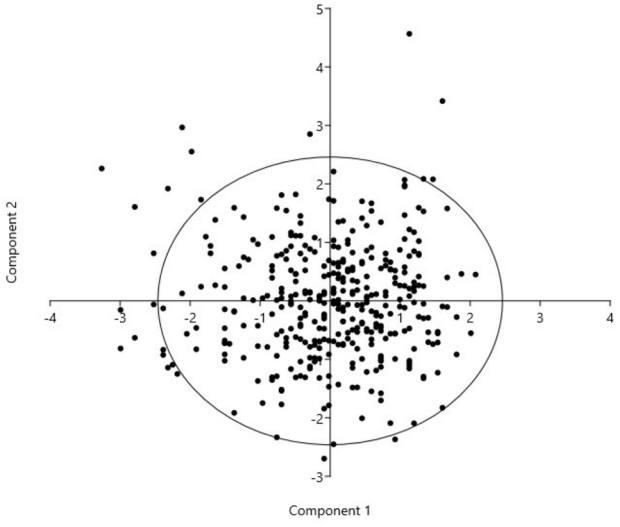

Figure 4. PCA plot on the five most prominent variables. Plot to analyze the most distinctive pattern (or clusters) (if any) among patient samples based on age, sex, hMSH2 & hMSH6 gene expression, and FGA values. The two displayed PC1 & PC2 axes covers 98.932 and 0.77107% variance respectively. Circle represents 95% confidence interval.

DISCUSSION

Mutations are prevalent in biological systems, with about 100,000 errors occurring during a single DNA replication (20). The MMR pathway is responsible for correcting some of these replication errors and, thereby, is critical for the effective functioning of the cellular processes by maintaining genome stability (6). A deficiency in MMR significantly hampers the ability of DNA to correct the mutations. Also, if left unrepaired, these mutations can drastically affect cellular homeostasis, even leading to cell death or tumor in many cases (21). MMR's functioning in glioblastoma is little known, though it is a downstream pathway to *MGMT*, the only significant known biomarker of GBM. MMR coordinates with *MGMT* to repair some DNA damage, which necessitates exploring the co-functional pathways to identify new biomarkers. It is essential to explore the correlation of MMR on genome alterations and check its efficacy as a potential prognostic marker using publicly available datasets. An analysis like this one may determine possible indications that require further *in-vitro* or *ex vivo* studies to help better understand glioblastoma (4).

We used the KPS, a scale that measures the everyday functional capabilities of cancer patients before surgery, to determine the severity of the effect of GBM on patients. An interesting observation is that though the KPS is very good in about seventy percent of the patients, with less than four percent having a low KPS, the disease-free months and overall survival of the patients are low, with 83.29% deceased by the end of 4 years. This potentially indicates the complexity and fatality of glioblastomas and emphasizes the need to explore this observation of how good KPS has no effect on disease-free and overall survival further.

We determined that there was little to no correlation between *hMSH2* and *hMSH6* gene expression with FGA. Although *hMSH6* mRNA and MSH6 protein have weaker positive correlations with FGA, the data indicate that, unlike hypothesized, *hMSH2* and *hMSH6* only affect the FGA to a lesser degree. Also, as highlighted from the results, this

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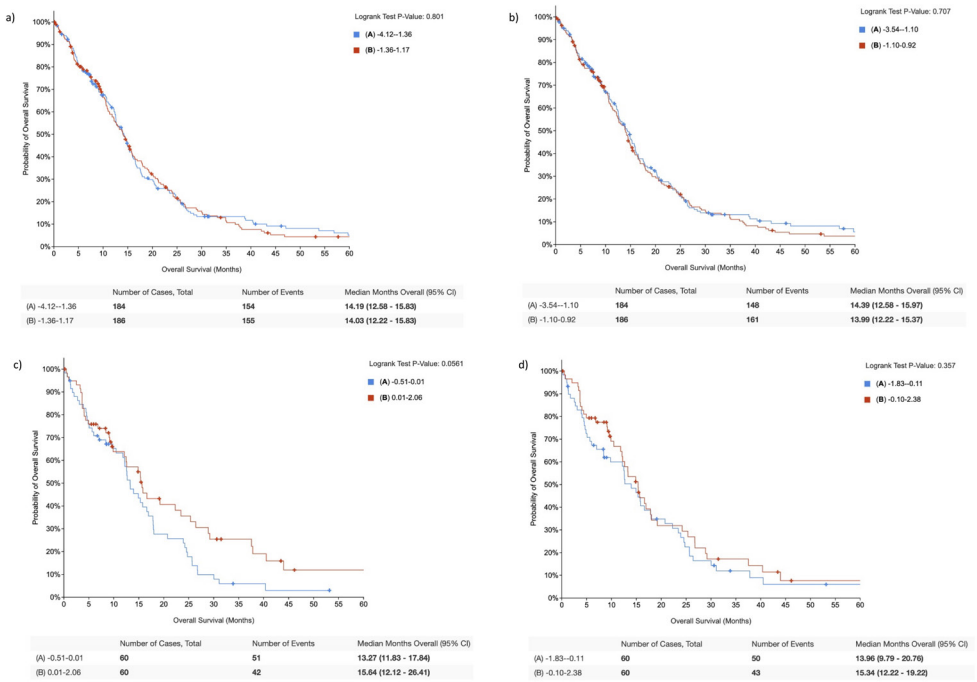

Figure 5. Kaplan-Meier plot demonstrates the non-significant impact of hMSH2 and hMSH6 with OSS. Plots depict the likelihood of surviving over a period of 60 months for gene expression and protein expression. Patients were split into two groups for each plot: low expression (below the median, group A) and high expression (above the median, group 2=B). **a**) Non-significant impact of high or low hMSH2 expression on Overall Survival Status of patients (p-value: 0.801). **b**) Non-significant impact of high or low hMSH6 expression on Overall Survival Status of patients (p-value: 0.707). **c**) Near significant impact of high or low MSH2 expression on Overall Survival Status of patients (p-value: 0.0561). **d**) Non-significant impact of high or low MSH6 expression on Overall Survival Status of patients (p-value: 0.357).

regulation might be sex-dependent—*hMSH6* has a significant correlation only in the case of male samples. Although this can be a case of misguided significant result due to the limited sample size in this cohort, it warrants the need for longitudinal studies. As anticipated, a total positive correlation could have demonstrated that the higher the expression of MMR genes, the lower the FGA, but this may be expected if the genes were functional and the relationship between their expression and FGA is empirical and well understood. Our study's result may suggest that both *hMSH2* and *hMSH6* may not dominantly be involved in the causation of glioblastoma. However, only a functional study could really indicate the underlying mechanisms. However, further studies are required to get more insights into this. This exploratory study just demonstrates the complexity and need to explore this pathway in the search for identifying biomarkers of value.

Overall, we found no significant difference in lower versus higher *hMSH2* or *hMSH6* expression on the OSS. This implies that the expression of these genes cannot accurately predict prognosis or survival. However, one cannot rule out the possibility of it being a potential biomarker. This *in silico* study could be useful to potentially explore the data and lead to new directions.

Limitations exist in this study that, if addressed, could lead to a more significant result. We relied on publicly available data from TCGA and cBioPortal with a limited patient sample size. Some of this data was incomplete and had to be cut down to be used for analysis; for example, only 120 samples were available for protein expression. Also, since this was an *in silico* analysis, we based our interpretation completely on correlation results. Hence, experimental studies analyzing the biological mechanisms to validate/disprove the causal relationship between the MMR pathway with cases of glioblastoma need to be performed. Another critical area of investigation can be large-scale studies to analyze the extent of expression difference between glioblastoma patients and healthy controls. Nonetheless, more comprehensive studies with more sample sizes could better enhance our understanding of the molecular biology of glioblastoma.

Thus, overall, we conclude that in our study, there is no statistically significant difference between *hMSH2* and *hMSH6* gene expression and FGA in glioblastoma patient samples. However, higher protein expression of MSH2 demonstrated a better OSS. Therefore, this study places emphasis on the need to better analyze other genes using publicly available datasets to determine their role in GBM. In specific, studies that analyze all the genes of MMR pathway need to be conducted with more dependent variables to better elucidate the actual role of MMR. Once such studies are performed, we might identify new relevant biomarkers for glioblastoma.

MATERIALS AND METHODS

Data Acquisition

A preliminary literature survey was performed using the NCBI-Pubmed to conduct this *in silico* study. The dataset was acquired from The Cancer Genome Atlas (TCGA) program, licensed publicly through the Genomic Data Commons (GDC) Portal (22). The experimental dataset TCGA-Glioblastoma Multiforme (Firehose Legacy, 2012) with 607 samples was chosen. The data file used from TCGA was mRNA expression

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microarray 'data_expression_all_sample_Zscores'. The clinical data for the patients were obtained from cBioportal, licensed by the National Institutes of Health (NIH) (20,23). The data file used from cBioportal was 'gbm_tcga_clinical_data'.

Data Processing

To prepare the dataset for analysis, the data was sorted by selecting data relevant to the hypothesis. The complete data initially consisted of 607 individuals. However, not all the original 607 patients had exclusive data needed for the hypothesis. After sorting for variables such as age, sex, FGA, OSS, and KPS, the total number of patients obtained was 366. Then the experimental data from TCGA (*hMSH2* and *hMSH6* gene expression) was matched with the clinical data for each patient. Protein expression data for both genes were only available for 120 patients. Finally, a separate protein data set was created for the analysis.

Data Analysis

The statistical analysis was performed using RStudio and PAST software (24). Values such as age, FGA, *hMSH2*, and *hMSH6* expression were categorized into two categories based on their median to determine if the higher or lower expression influenced survival. Further, the Spearman correlation test was used to calculate the correlation between the FGA and *hMSH2/hMSH6* expression. We determined significance by using a significance level of $p<0.05$. Kaplan-Meier plots were plotted to analyze whether the expression of these two genes impacts OSS (25). These steps were repeated for the respective proteins MSH2 and MSH6.

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