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MicroRNA Expression in Low- and High-Grade Gliomas in Pediatric Patients and Correlation with Matrix Metalloproteinase Expression

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Abstract

Study background: Over the past years, the number of patients diagnosed with glioma has increased. Glioma is the most widespread pediatric malignancy of the brain and a high-grade tumor is associated with a dismal prognosis. Members of the matrix metalloproteases (MMP) enzyme family (especially MMP-2 and MMP-play an important role in the degradation of the extracellular matrix (ECM) a requirement for disease progression, in addition to the miRNAs play a vital role in regulating "cancer hallmarks".

Aim: Our aim in this study was to detect the expression level of MMP2 and MMP9 in high-grade glioma and low-grade glioma to find the differential expression in two different subtypes and to find the correlation of MMP and progression of disease and to identify any relationship between miRNA and MMP expression in gliomas.

Methods and finding: We determined the expression of all the miRNAs and mRNAs for the (MMP2 and MMP9) in over 20 high grade glioma (HGG) and 20 low grade glioma (LGG) in paraffin-embedded (FFPE) using Quantitative polymerase chain reaction (qPCR).

Result: Seven miRNAs showed high expression specifically in HGG compared to LGG with significant differential expression (p-value ≤ 0.05).

Conclusion: The mRNA for the MMP-2 and MMP-9 were highly expressed in HGG than in LGG, although the difference did not reach the statistical significance. The main limitations of this study are the small sample size of the patients and further work is needed to investigate the relativeness of our work to the clinical side.

Keywords: Matrix metalloproteinases; Paediatric brain tumours; MicroRNA

Introduction

The glioma tumor is the most widespread malignancy of the brain worldwide. The World Health Organization (WHO) classifies gliomas into four grades (I–IV) based on the level of malignancy: low grade glioma (LGG) (WHO grades I–II) and high-grade glioma (HGG), which is further classified into anaplastic astrocytoma (AA, WHO grade III) and glioblastoma multiforme (GBM, WHO grade IV). The most common form of glioma is GBM, with a prevalence of 10000 new cases in the US. In Egypt, gliomas represent 37.3% of CNS tumors, while GBM accounts for 33.8% of all gliomas [1]. HGG is the most aggressive form of glioma due to its rapidly infiltrating growth pattern, and the mean survival is only 15.2–18 months. By contrast, LGG is associated with a good prognosis, with a 5-year survival rate of 30-70%. The poor prognosis for HGG greatly emphasizes the need to understand and identify prognostic biomarkers that can discriminate HGG from LGG to improve patient survival [2-6].

The matrix metalloproteases (MMPs) enzyme family, which plays an important role in the degradation of extracellular matrix (ECM). Accumulating evidence supports an association between MMPs and pathological conditions such as cancer cell invasion and metastasis, imparted by the ability of these enzymes to degrade the ECM of tumor cells. Previous studies have reported that MMPs participate in the invasive and aggressive behavior of glioma malignancies, and the gelatinase group, including MMP-2 and MMP-9, has drawn the most attention [7,8]. Previous studies showed that increased expression of MMP-2 and MMP-9 was significant with the degree malignancy in gliomas, which plays a role in metastasis and invasiveness of the tumor, they also showed that MMP-9 is more intense and elevated when compared to the MMP-2. Another study shows association between the MMP-9 with malignancy in glioma and formation of the angiogenesis [9,10]. Studies shows strong relation between the MMP-9 and the establishment of the angiogenic process, the switch requires proteolytic release of the vascular endothelial growth factor (VEGF) by MMP-9, followed by formation of vascular network that ease the tumor spread to various sites [11-13]. Another potential class of biomarkers that is receiving increasing attention is microRNAs (miRNAs). These are members of a large family of non-coding short single stranded RNAs ~22 nucleotides (nt) in length (range 18-25 nt) that bind to the 3' untranslated region (UTR) of protein-coding mRNAs and regulate gene expression. The miRNAs are involved in a variety of biological processes, including cell development, proliferation, differentiation, apoptosis, and cell cycle control. Various studies have shown a correlation between abnormal miRNA expression and the development

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and pathogenesis of the different types of cancers, including GBM. Consequently, miRNAs are finding uses as biomarkers for cancer diagnosis and prognosis. Several studies have shown that one role of miRNAs is to regulate MMP gene expression during the progression of cancer, as impairment of miRNA processing is associated with increased expression of MMP-2 and MMP-9 [3,14-17].

The discovery of new biomarkers therefore appears to be vital for improving the poor prognosis of patients with GBM. Further investigations are now being conducted with the aim of discovering new and effective therapeutics for GBM based on biomarkers. Consequently, the understanding of the role of MMPs and their correlation with miRNA expression in GBM might provide advances in prognosis and treatment, due to the key role of miRNAs as post-transcriptional regulators. The involvement of miRNAs is recognized in a variety of biogenesis and biofunctional processes, and miRNAs are now routinely used in the management of patient with cancer [18-20].

In the present study, our aim was to find the differential expression of MMP2 and MMP9 in HGG compared to LGG and to find the possible relationship between MMPs and miRNAs expression. The goal was to identify candidate miRNAs that regulate MMPs in brain tumors, which may help to understand the role of MMP in HGG and how they can be regulated by miRNAs.

Materials and Methods

Patients and specimens

This study was retrospective study and approved by the Children's Cancer Hospital (CCHE) -57357-Egypt. Informed consents were obtained from all subjects selected for participation in the study. A total of 40 formalin-fixed, paraffin-embedded (FFPE) glioma tissue specimens were obtained from patients who underwent surgical resection in the Department of Neurosurgery at CCHE between 2008-2015. All specimens were obtained directly after surgical resection and were processed by the pathology lab in a paraffin block. The sample collection was approved by the institutional review board (CCHEIRP #12-2014). All samples were kept anonymous and were handled according to ethical standards.

A total of 30 females and 10 males were enrolled in this study, with a median age of 8 years (range 3–16 years). Of the 40 gliomas, 20 were classified as LGG (20 pilocytic astrocytoma [WHO grade I], 20 were HGG (3 anaplastic astrocytomas [WHO grade III] and 17 glioblastomas [WHO grade IV]) according to the WHO classification for tumors of the CNS [21]. A total of 82 miRNAs were identified between HGG and LGG in a previous study, from which the highest statistically significant miRNAs between HGG and LGG were selected for the further investigation in current study [22].

RNA extraction

Total RNA was extracted from 40 samples of formalin-fixed, paraffin-embedded brain tumor tissue (cut into sections 5-10 each 5μm thick) using a miRNeasy FFPE Kit (Qiagen, Germany) according to the manufacturer's instructions. The total RNA concentration was measured using a Nanodrop instrument (ND 1000 - Thermo Scientific, USA).

RT-Reverse transcription and qualitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was converted to complementary DNA (cDNA) using a miScript II RT Kit (Qiagen, Germany). Each reaction tube contained miRNA equivalent to 10 ng of total RNAs, 200 nM miRNA primer (Invitrogen), 200 nM universal primer (Invitrogen), and 1 × SYBR Green (miScript SYBR Green PCR Kit (Qiagen- Germany) in a total of 10 μl. The real-time cycler was programmed as follows: initial denaturation 10 min. at 95 °C, followed by 95°C for 15 sec, 55°C for 30 sec, and 72°C for 30 sec (repeated for 40 cycles). The most significant miRNAs expressed in LGG and HGG brain tumors were selected from previous study published in the same lab for testing in this study and were obtained from Invitrogen. Mature miRNAs were designed and are listed in Supplementary Table 1 [22].

The mRNA reactions for MMP-2 and MMP-9 contained 1.5 μl (~100 ng) template mRNA and 200 nM forward and reverse primer in a total volume of 10 μl. The real-time cycler was programmed as follows: for MMP-2, initial denaturation 15 min at 95°C, followed by 94°C for 15 sec, 58°C for 30 sec, and 70°C for 30 sec (repeated for 40 cycles); for MMP-9, initial denaturation 15 min at 95°C, followed by 94°C for 15 sec, 55°C for 30 sec, and 70°C for 30 sec (repeated for 40 cycles). The reactions were conducted in a real-time cycler (Applied Biosystems Quant Studio™ 6 Flex Real-Time). The RT-PCR primers for MMPs were designed as follows: MMP-2 forward (Invitrogen), 5'-AGCTCCCGGAAAAGATTGATG-3' and reverse (Invitrogen), 5'CAGGGTGCTGGC TGA GTAGAT-3'; MMP-9 forward (Invitrogen), 5'-CCTGGA GACCTGAGA ACCAATC -3' and reverse (Invitrogen), 5'-GATTTCGACTCTCCACGC ATCT-3'. The reference gene, glyceraldehyde-3-phosphatedehydrogenase (GAPDH), was amplified and used as an internal control to estimate the integrity and amount of cDNA using the GAPDH primers, forward: 5' TGA AGG TCG GAG TCA ACG GAT TT-3' and reverse: 5' GCC ATG GAA TTT GCC ATG GGT GG -3'.

Quantitative polymerase chain reaction (qPCR) statistical analysis

The relative expression level of the miRNAs was normalized using the dCT method as follows: All CT values greater than or equal to 35 were replaced with 35 before calculating the mean of the remaining CT values. The mean CT value was calculated for each sample is

$$\Delta\Delta Ct = \Delta Ct\ sample - \Delta Ct\ average\ normal\ samples$$

[23].

The ΔΔCt and fold changes were considered based on the subsequent equations: $\Delta\Delta Ct = \Delta Ct\ sample - \Delta Ct\ average\ normal\ samples$

$$Fold\ change = 2^{\wedge} - \Delta\Delta Ct$$

The relative expression changes in MMP-2 and MMP-9 were determined as previously explained for the miRNAs. Lower dCT scores represent higher expression [24]. The expression data were analyzed statistically using the Multi-Experiment Viewer (MeV_4, Boston, MA, USA) software package.

Statistical analysis

All data were analyzed using SPSS software version 22 for Windows (SPSS Inc., IL, and USA), Graph Pad Prism 7 (Graph Pad Software Inc., CA, USA), and Multi experiment Viewer (MeV v 4.9). We identified the cellular gene targets for miRNAs using bioinformatics searching tools available online, such as TargetScan, which predicts the targets of miRNAs. Numerical data were expressed as mean ± standard deviation (SD), median, and range. The Kolmogorov-Smirnov Z test was used to determine the equality of normalization defined by the level of skewness and kurtosis. Numerical variables between the study

groups were compared using Student's t-test for independent samples when comparing two groups that were normally distributed and the Mann-Whitney U test for independent samples when the data were not normally distributed. A p value ≤ 0.05 was considered significant. Qualitative data were expressed as frequency and percentages. The chi-square test (χ^2) or Fisher's exact test was used to examine the relationship between qualitative variables. The Pearson correlation coefficient for continuous variables and the Spearman correlation for ordinal variables were used to test the presence of a relationship between the miRNAs and MMPs. Life-table estimates were calculated using the Kaplan-Meier method and differences between curves were tested for statistical significance using the log-rank test (Tables 1 and 2).

Results

Patient characteristics

The samples used in this study were obtained following post-surgical excision and revised at the pathology department of the Children's Cancer Hospital-57357 (CCHE). In a previous study, we identified the differential expression pattern of 82 miRNAs between HGG and LGG. We selected miRNAs with the highest statistical significance between HGG and LGG for the current study and we validated the significant changes between these miRNAs and the subtypes under investigation. A correlation was noted between gene and protein expression of both MMP-2 and MMP-9 in pediatric neoplastic brain tissues (gliomas) and the survival of 20 patients with LGG (World Health Organization (WHO grade I) and 20 patients with HGG (WHO grade III–IV). The comparative Ct (ΔΔCt) method was used to determine the fold changes of expression of each miRNA in the tumor samples relative to LGG.

Clinico-pathological features	Low Grade Glioma (n=20)	High Grade Glioma (n=20)
Gender		
Female	17 (85%)	13 (65%)
Male	3 (15%)	7 (35%)
Grade		
I	20 (100%)	0 (0.0%)
III	0 (0.0%)	3 (15%)
IV	0 (0.0%)	17 (85%)
Age (in years)		
Mean	7.7	8.6
Median	7	9
Range	3-15	4-16
Age Category		
>1 year and <10 years	15 (75%)	15 (75%)
≥ 10 years	5 (25%)	5 (25%)
Tumor size		
≤ 5 cm	15 (75%)	11 (55%)
>5 cm	4 (20%)	8 (40%)
NA	1 (5%)	1 (5%)
Metastasis at Presentation		
Yes	0 (0.0%)	2 (10%)
No	20 (100%)	18 (90%)
Risk		
High	0 (0.0%)	20 (100%)
Low	20 (100%)	0 (0.0%)
Tumor Extent		
STR	2 (10%)	8 (40%)
GTR	18 (90%)	12 (60%)
Survival Status		
Alive	19 (95%)	5 (25%)
Dead	1 (5%)	15 (75%)

Table 1: The distribution of the clinico-pathological characteristics of malignant glioma patients enrolled in the present study.

miRNAs	Correlation Coefficient	MMP-2	MMP-9
mir_19a-5p	r	0.567**	0.564**
	p-value	0.000	0.000
mir_19b-5p	r	0.423**	0.449**
	p-value	0.007	0.004
mir_24-1-5p	r	0.531**	0.584**
	p-value	0.000	0.000
mir_26a-5p	r	-0.501**	-0.499**
	p-value	0.001	0.001
mir_27a-5p	r	0.443**	0.514**
	p-value	0.004	0.001
mir_584-5p	r	0.662**	0.616**
	p-value	0.000	0.000
mir_527	r	0.359*	0.220
	p-value	0.023	0.173

Table 2: The following table is showing overall, 7 miRNAs with a statistical significance relationship with the MMP-2 and MMP-9.

Matrix metalloproteases expression in high-grade and low-grade glioma groups

Previous studies have implicated the gelatinase group (MMP-2 and MMP-9) in invasion and aggressiveness of HGG. Therefore, in the present study, we tested the gelatinase expression in our cohort with the aim of identify potential biomarkers. Our comparison of MMP-2 and MMP-9 expression between HGG and LGG groups, relevant differential expression only for MMP-9 (p-value ≤ 0.068), which was more highly expressed in patients with HGG than LGG, although this difference did not reach statistical significance. By contrast, MMP-2 showed a similar expression pattern in both HGG and LGG tumors. MMP-2 and MMP-9 expression patterns are shown in Figure 1.

Identification of candidate microRNA signatures between pediatric HGG and LGG Pediatric brain tumors

In total, 82 miRNAs were compared between the HGG and LGG groups. This comparison indicated a significant difference ($p \leq 0.05$) in the occurrence of 7 miRNAs between the two glioma types as shown in Supplementary Tables 2 and 3. Has-miR-527, miR-19b-2-5p and miR-26a-5p showed high expression in HGG compared to LGG, miR-19a-5p, miR-24-1-5p, miR-27a-5p and miR-584-5p were decreased in HGG compared to LGG as shown in Figure 2. We hypothesized that deregulated miRNAs have a role in the poor prognosis in patients with HGG.

MMPs and microRNAs expression in glioma cells

Correlation between the MMP-2 and MMP-9 and miRNA expression was assessed using spearman's correlation for non-linear relationships and Pearson correlation for linear relationships. A high correlation (>0.5) value was observed, as shown in Supplementary Figure 1. We concluded that MMP-2 and MMP-9 might therefore represent target genes for the miRNAs.

Discussion

GBM is the most fatal central nervous system malignancy and has a dismal prognosis regardless of treatment [25,26]. To ensure improvement of the poor prognosis of patients with GBM in the future,

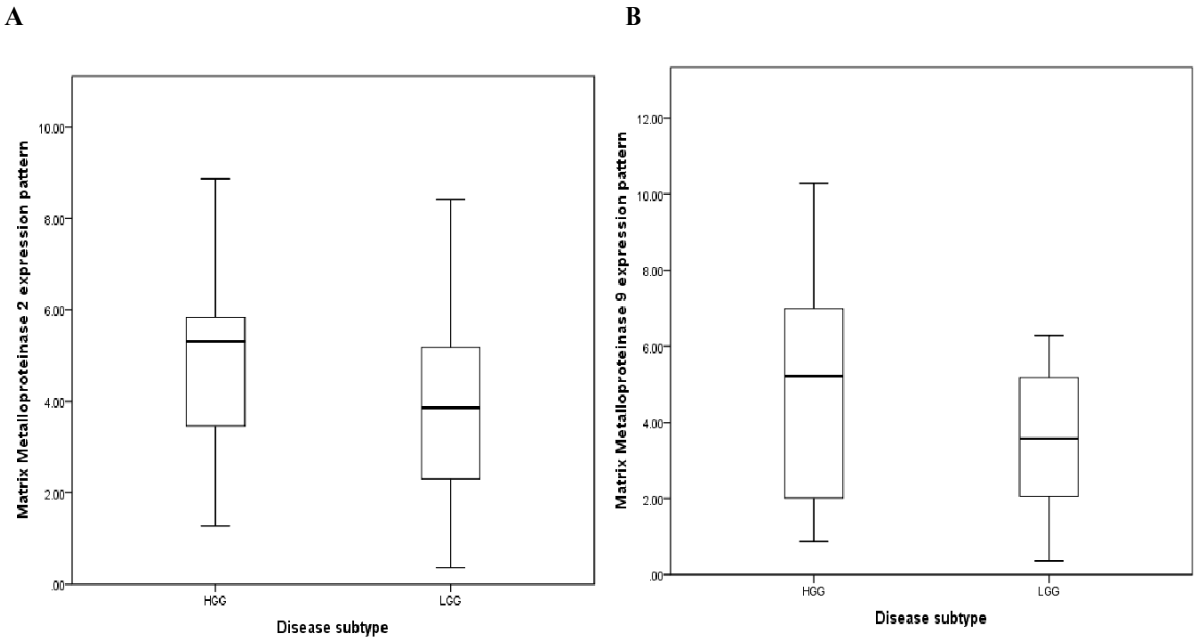

Figure 1: Box plot showing (**A**) The delta-CT values of the MMP-2 in HGG compared to LGG, (**B**) The delta-CT values of the MMP-9 in HGG compared to LGG.

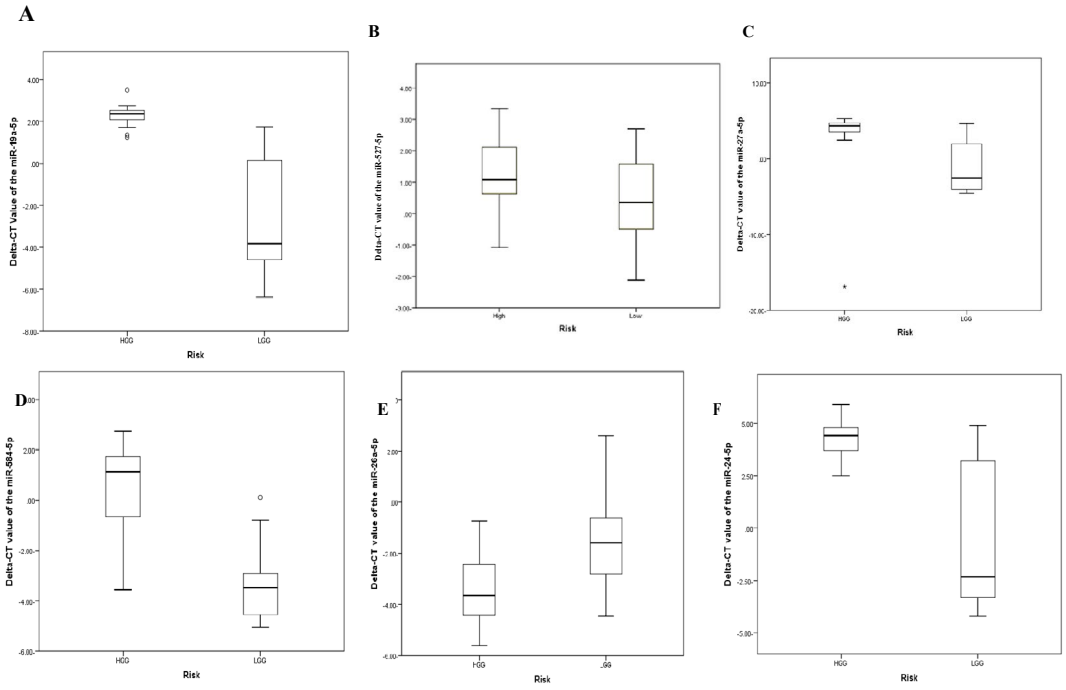

Figure 2: Box plot showing the deregulated miRNAs in HGG compared to LGG. Figure (A-F) shows the delta-CT values with high expression of miR-19a-5p, miR-527-5p, miR-27a-5p, miR-584-5p, miR-26a-5p and miR-24-5p in HGG compared to LGG.

more investigations are needed to identify effective therapeutics based on biomarkers. MiRNA expression in GBM might provide advances in prognosis and better treatments, because of the main role of miRNAs post-transcriptional regulators. At present, the involvement of miRNAs is implicated in a variety of biogenesis and bio-functional processes, but no role for the microRNAs profiling has yet been published for GBM due to difference in study design [18-20]. In the present study, to identify differentially expressed MMP-2 and MMP-9, we first compared RNA samples of the HGG group to the LGG group using qPCR moreover, in order to predict the miRNA-gene target, we compared MMP-2 and MMP-9 to 82 miRNAs, from which 7 miRNAs that showed significantly different expression pattern in

HGG compared to LGG correlated significantly with the MMP-2 and MMP-9. Three miRNAs showed high expression pattern in HGG when compare to LGG, while 4 miRNAs showed decrease in expression in HGG compared to LGG group.

The deregulated miRNAs that could differentiate the gliomas, were miR-26a-5p, miR-19b and miR-527 which were highly expressed in HGG compared to LGG. The high expression of these miRNAs was correlated with tumor grading as determined in previous studies. Moreover, Huse et al., reported that miR-26a was overexpressed at the RNA level and detected amplification was detected at the DNA level, which resulted in decreasing the PTEN which dramatically dropped the disease free-survival. Furthermore, miR-19 (miR-19a and miR-19b) was reported to enhance the proliferation and invasion in glioma, we report only high expression of miR-19b and decrease in miR-19a in in HGG compared to LGG [27-43]. We believe, small sample size might also explain why some of our data failed to reach statistical significance.

The key signature of HGG, as indicated by previous studies, was the downregulation of the following miRNAs in HGG compared to LGG; miR-27a-5p and -miR-584-5p were decreased in HGG compared to LGG**.** Similar studies showed that the tumor suppressor miR-27a-5p were significantly downregulated in various cancers and this was associated with disease progression. On the other hand, earlier studies showed an overexpression of the miR-24-1-5p, were inhibition of this miRNA inhibits the proliferation and invasion, in current study we report downregulation of miR-24-1-5p Moreover, under expressed of the miR-584-5p associated with probability of low survival in neuroblastoma [44-52].

The MMPs are known to have an important role in cancer invasion and metastasis due to them for its ability to degrade the extracellular matrix (ECM) between cells [53-55]. MMP-9 was upregulated in HGG and its expression correlated with the glioma WHO grade. Our use of bioinformatics tools allowed further exploration of the role of miRNAs in glioma genesis and the mechanism by which they control MMP-2 and MMP-9. We report in this study, 7 miRNAs with positive relation with the MMP-2 and MMP-9. MMP-2 was reported as a direct target for the miR-26a-5p, both were overexpressed in lymph node metastasis tumor tissue and known to enhance cell migration and invasion abilities [33,56]. Moreover, under expression of the miR-584-5p was contrariwise with the expression of the MMP-14, in current study we report MMP-2 and MMP-9 can be considered as direct targets for the miR-584-5p.

Conclusion

In the present study, we tried to find the difference in the expression of MMP-2 and MMP-9 in HGG and LGG and to correlate their expression with miRNAs. We believe this study can be used as a pilot study to understand one of the most aggressive central nervous system tumors in Egyptian population and that our findings can assist in developing the understanding of aggressiveness of HGG.

Author Contributions

D.Y. and M.T. conceived and designed the experiments; D.Y. performed the experiments; D.Y. performed the data analysis; D.Y. contributed reagents/materials/analysis tools; D.Y. wrote the paper." H.T as a pathologist revised the slide and diagnosed the glioma patients. M.T, H.T, M.F, S.G and E.A revised the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

Journal Scope

We believe this journal is an appropriate venue for publishing our manuscript as it focuses on biomarkers and the scope of our manuscript focuses on exploring biomarkers in Egyptian patients diagnosed of "high-or low-grade glioma" brain tumors.

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