ANTICANCER RESEARCH *44*: 5199-5205 (2024)
doi:10.21873/anticanres.17346

LET-7a and *LET-7b* as Potential Signatures for Glioma Recurrence, Regardless of WHO Tumor Grade

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Abstract. *Background/Aim: Micro RNAs (miRs; miRNAs) are small, non-coding RNA segments that influence gene expression. To do this, they bind to corresponding mRNA segments and inhibit the subsequent protein translation. Currently, about 200 transcripts are thought to be regulated by a single miRNA. Dysregulated miRNAs can act as tumor promoters as well as tumor suppressors. The aim of this study was to verify miR-LET-7a and miR-LET-7b as a signature for glioma recurrence detection by analyzing their expression in glioma tissue. Materials and Methods: Only patients with two or more recurrences of glioma (n=25) were included in the study (tissue samples, n=89). Tissue was obtained during neurosurgical procedures and shock-frozen in liquid nitrogen. The patient cohort (female:male=12:13) had an average age of 35 years when first diagnosed (primary tumor: 13 WHO grade II, 7 WHO grade III and 5 WHO grade IV) and 48 years in the fifth relapse. Quantitative real-time polymerase chain reaction was performed to analyze miRNA expression. miR-151a-3p was used as an internal reference. Results: Expression of miR-LET-7a and miR-LET-7b was significantly lower in the first recurrence than in the primary tumor. The expression of miR-LET-7a and -7b significantly decreased with increasing tumor grade. The strongest down-regulation was found between WHO grade III and IV. In paired samples in which tumor grade did not change between the primary and the first recurrence, the expression of both miRNAs remained significantly lower in the recurrent than in the primary tumor. Conclusion: miR-LET-7a and miR-LET-7b represent a potential signature for glioma recurrence regardless of WHO grade.*

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Key Words: miRNA, gene signature, glioma, LET-7.

Gliomas account for the largest proportion of neuroepithelial tumors. Classified into four grades by the 2016 edition of the World Health Organization Classification of Tumors of the Central Nervous System (1), grade IV glioblastoma is the most common and aggressive form, accounting for 59% of all gliomas (2). Despite current treatment options consisting of neurosurgical tumor resection combined with chemoradiotherapy, the median survival time is only about 15 months (3).

Molecular alterations for reliable tumor classification and patient's prognosis are becoming more important. Alongside already established biomarkers, such as O^6-methylguanine-DNA methyltransferase methylation, 1p/19q co-deletion, and mutation of isocitrate dehydrogenase 1 or 2 gene (4), so-called microRNAs (miRs; miRNAs) represent potentially new markers for diagnosis, therapy and monitoring of brain tumors (5).

miRNAs are small, non-coding RNA segments with a length of approximate 20 nucleotides that influence gene expression as they bind to corresponding mRNA segments and inhibit the subsequent translation of proteins. In this way, a single miRNA can influence about 200 different transcripts, each of which is involved in different signaling pathways (6, 7).

When dysregulated, miRNAs can act as both tumor promoters and tumor suppressors. By controlling cellular processes, such as angiogenesis, proliferation or invasion, they play an important role in metastasis (8). To date, numerous miRNAs whose expression was either down- or up-regulated have been identified in glioma tumor tissue (5). Moreover, signatures consisting of multiple miRNAs have already been successfully verified which may help in the diagnosis of gliomas as well as in the prediction of prognosis in the future (9-11). Niyazi *et al.*, for example, created a signature using

Table I. *Demographic and clinicopathological characteristics of patients included in the study.*

Tumor sample	Number of samples	Average age at diagnosis, years	Sex, n		Preoperative therapy, %		Tumor location				
			Male	Female	CT	RT	Front	Temp	Pariet	Occ	Cereb
Primary	25	35	13	12	0	0	20	3	1	1	1
Relapse 1	23	39	13	10	48	70	16	3	3	1	1
Relapse 2	23	42	11	12	57	78	19	3	1	1	1
Relapse 3	13	44	6	7	77	100	11	3	1	0	0
Relapse 4	5	43	2	3	80	100	5	1	0	0	0
Relapse 5	1	48	0	1	100	100	1	0	0	0	0

Cereb: Cerebellar; Front: frontal; Occ: occipital; Pariet: parietal; Temp: temporal. When the location of tumor was frontotemporal, the sample was counted at both locations.

miR-LET-7a, miR-LET-7b, miR-125a-5p, and *miR-615-5p* with the aim of being able to predict the therapeutic outcome of patients with glioblastoma. Therefore, high- and low-risk patient groups based on the expression level of these miRNAs were compared, resulting in a significantly shorter overall survival for the high-risk group (11).

Further studies also identified *miR-LET-7a* and *miR-LET-7b* as tumor suppressors in gliomas, being down-regulated in tumor compared to control tissue. Up-regulation of both miRNAs in glioma cell lines slowed tumor growth and thus makes them potential candidates for targeted therapy in the future (12-14). A correlation between tumor grade and expression levels of both miRNAs has also been demonstrated as the expression was lower in high-grade (WHO III and IV) than in low-grade (WHO II) tumors. In addition, lower expression of *miR-LET-7a* and *miR-LET-7b* in these tumors was associated with a poorer prognosis (12, 15). In 2018, another study investigated the expression levels of both LET-7 miRNAs in the first recurrence of glioblastoma in patients compared to the primary tumor. The expression was found to be significantly lower in the recurrent than in the primary tumor (16).

However, an analysis of the expression of *miR-LET-7a* and *miR-LET-7b* in the longitudinal course, *i.e.* beyond the first recurrence, has not been performed. In addition, only glioblastoma samples and thus high-grade tumors, were examined in the 2018 study. The aim of this study was therefore to verify *miR-LET-7a* and *miR-LET-7b* as a potential signature for relapse beyond the first recurrence and, more importantly, as a marker of recurrence of gliomas of any WHO grade.

Materials and Methods

Patients and *tissue samples*. Glioma tissue from 25 patients treated at the Department of Neurosurgery at the University Hospital of Cologne between 1991-2020 was investigated. For the selection of suitable tissue samples for the longitudinal study, it was a prerequisite that each patient had both primary glioma and at least two further recurrences (see Table I). A total of 89 tissue samples were examined, which were divided into grade II, III, and IV (see Table II) according to the 2016 WHO classification (1, 17).

Table II. *Distribution of primary tumor and recurrences among World Health Organization (WHO) grades II-IV.*

Tumor sample	Number of samples	WHO grade		
		II	III	IV (Glioblastoma)
Primary	25	13	7	5
Relapse 1	23	4	12	7
Relapse 2	23	1	14	8
Relapse 3	13		7	6
Relapse 4	5		4	1
Relapse 5	1		1	

All tissue samples were collected during neurosurgical procedures at the University Hospital of Cologne, shock-frozen in liquid nitrogen and stored long-term at −80˚C.

The use of the samples for scientific purposes was according to the Helsinki declaration of ethical requirements and was approved by the local Ethical Committee of the University of Cologne (Application No. 03-170).

RNA isolation and *cDNA synthesis*. miRNAs were isolated from 30 mg of tumor tissue using miRNeasy Tissue Cells Advance Kit (Qiagen, Hilden, Germany) and Tissue Lyser LTII. Reverse transcription was then performed using miScript LNA RT Kit (Qiagen) according to the manufacturer instructions with a reaction volume of 10 μl and 10 ng of RNA per sample. The RNA was quantified and purity measured using an Infinite® 200 PRO (Tecan, Männedorf, Switzerland) and then diluted to 5 ng/μl. In addition, an RNA Spike-In Kit (Qiagen) was used as a quality control for miRNA isolation, cDNA synthesis and quantitative real-time polymerase chain reaction (qPCR).

qPCR. Real-time PCR was performed using a miRCURY LNA SYBR Green PCR Kit (Qiagen) with the Rotor Gene-Q Real-Time PCR System (Qiagen) according to the protocol provided. The

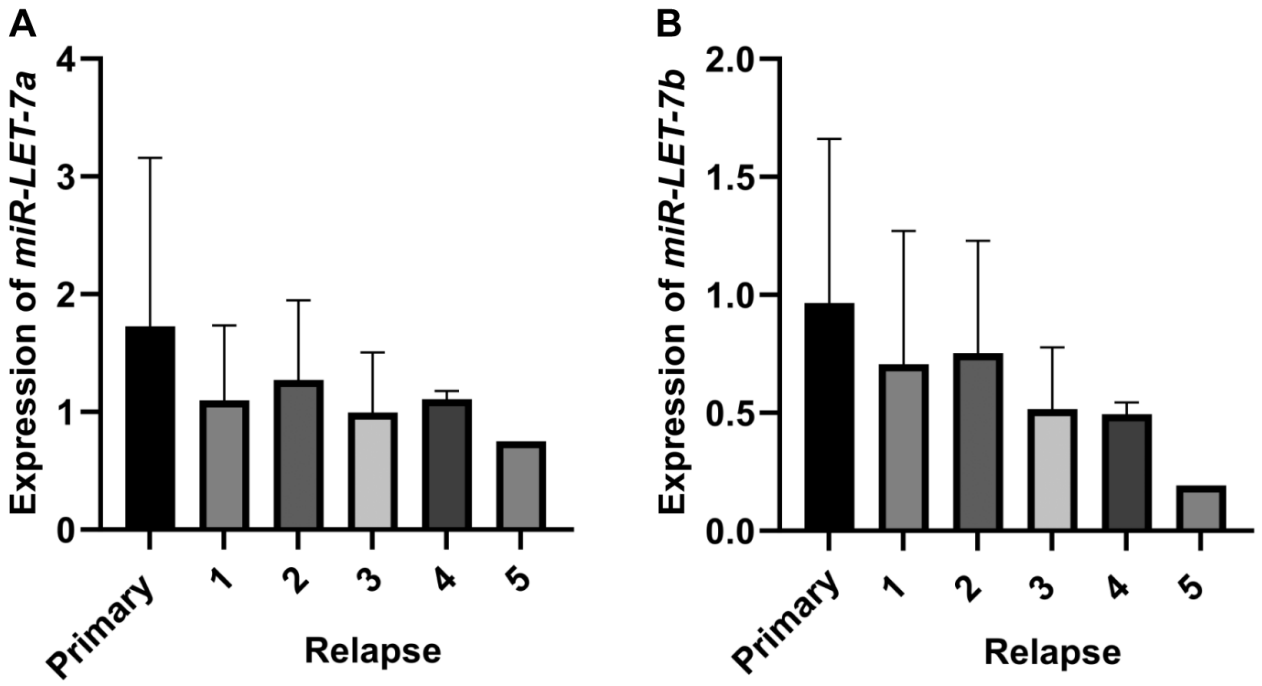

Figure 1. *Illustration of the expression of miR-LET-7a (A) and miR-LET-7b (B) in the primary tumor and up to and including the fifth relapse.*

following program was used with a reaction volume of 20 μl for 50 cycles: 95˚C for 2 min, 95˚C for 10 s, and 56˚C for 60 s. The manufacturer's instructions originally specified a reaction volume of 10 μl, but we were unable to regenerate results with this until we doubled the volume of each reagent. Before examining the tissue samples in detail, a reagent pool was created from different cDNA samples to generate standard curves for each primer. Primers *miR-LET-7a-5p* and *miR-LET-7b-5p* were tested; *miR-151a-3p* served as internal reference (miRCURY LNA miRNA PCR Assay; Qiagen). All qPCR reactions were carried out twice. The relative expressions of the miRNAs were calculated using the Rotor Gene Q Series Software (Qiagen), as were the melting curves after each run to confirm amplification specificity.

Statistical analysis. GraphPad Prism 9.0 (GraphPad Software Inc., La Jolla, CA, USA) was used for all statistical analyses.

For the longitudinal expression analysis of *miR-LET-7a* and *miR-LET-7b,* and for comparison of expression by WHO grade, the Kruskal–Wallis test was applied. For analyses of paired samples, as in the study of expression behavior in the primary tumor compared with the first relapse, the Wilcoxon signed-rank test was performed. The comparison of miRNA expressions between high- and low-grade tumors was analyzed by Mann–Whitney test. Recurrence-free survival was analyzed using Kaplan–Meier curves and the Mantel–Cox test was performed to compare the curves of recurrence-free survival. To measure recurrence-free survival, the period between the day of surgery for the primary tumor and surgery for the first recurrence was determined. In parallel, the difference between the expression values of *miR-LET-7a* and *miR-LET-7b* in the primary tumor and the first recurrence was calculated. After calculating the mean value from the expression rates of both miRNAs, patients with *miR-LET-7a* or *miR-LET-7b* expression above the mean value were classified as having high expression, patients with values below the mean value were classified as having low expression. Results were considered to be statistically significant when $p<0.05$.

Results

Firstly, the longitudinal expression of *miR-LET-7a* and *miR-LET-7b* from primary tumor until fifth relapse was analyzed (Figure 1). The number of patients in each group and the WHO classification of the tumor samples are shown in Table II. For both *miR-LET-7a* and *miR-LET-7b,* a trend for decreasing expression was found in the longitudinal course of the disease. When comparing the primary tumor with the first relapse, expression of *miR-LET-7a* and *miR-LET-7b* was significantly lower in the latter (Figure 2). Even if the tumor grade stayed stable, a lower expression in the first relapse was found, showing that this finding occurred independently of malignancy of the tumor.

We next wanted to know whether expression of *miR-LET-7a* and *miR-LET-7b* is influenced by tumor grade. Our results show significantly lower *miR-LET-7a/b* levels with higher tumor grades. This was true for low- *versus* high-grade tumors and also when comparing WHO grades II to IV (Figure 3). The analysis of *miR-LET-7a* showed a significantly lower mean expression in the high-grade

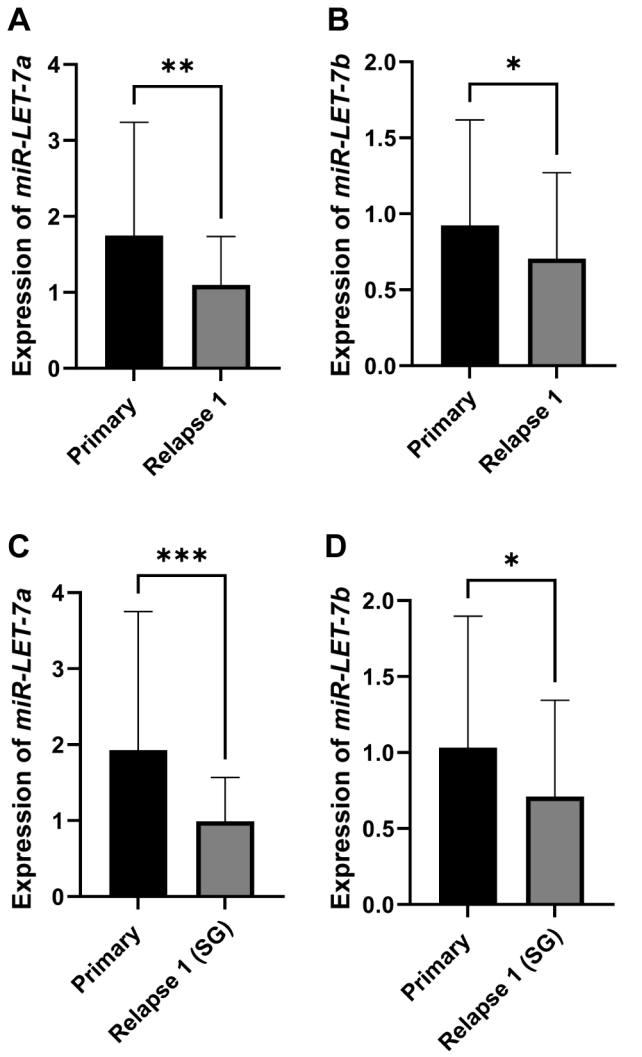

Figure 2. *Expression of miR-LET-7a and miR-LET-7b comparing (A) the first recurrence with the primary tumor and (B) primary tumor and recurrence of the same tumor grade (SG) (WHO II: n=4, WHO III: n=6, and WHO IV: n=5). Significantly different at: *p<0.05, **p<0.01, and ***p<0.001.*

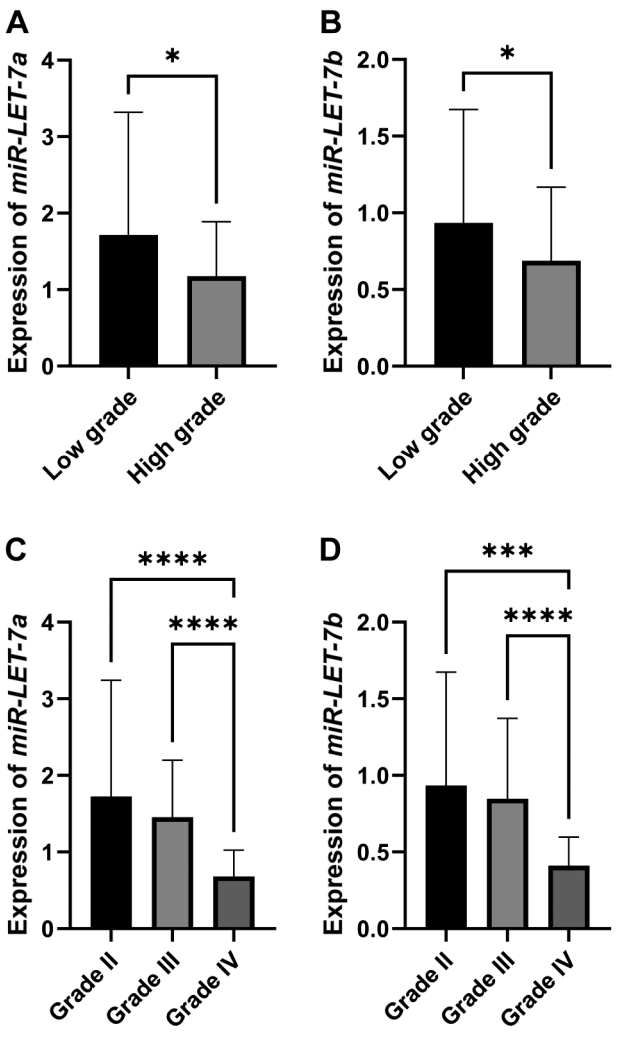

Figure 3. *Expression of miR-LET-7a and miR-LET-7b depending on the 2016 WHO classification of tumor grade. (A) Expression compared in low-grade (II) and high-grade (III+IV) tumors. (B) Expression according to tumor grade. Significantly different at: *p<0.05, ***p<0.001 and ****p<0.0001.*

tumors compared to low-grade tumors ($p<0.05$, low grade: 1.715±1.604 *vs*. high grade: 1.175±0.715) (Figure 2A). The same pattern was found for *miR-LET-7b* ($p<0.05$, low grade: 0.934±0.740 *vs*. high grade: 0.688±0.480) (Figure 2B).

Thus, miR-LET-7a showed the lowest expression in WHO grade IV compared to grade II and III. The strongest reduction in expression was observed between grade III and IV.

Lastly, recurrence-free survival according to high and low *miR-LET-7a* and *miR-LET-7b* expression was evaluated. There was a tendency for longer recurrence-free survival in patients with high *miR-LET-7a* expression compared to patients with low expression (Figure 4).

Discussion

Gliomas constitute the largest proportion of malignant primary tumors of the brain and central nervous system, accounting for nearly 80% (2). It is known that dysregulation of miRNAs plays a significant role in both tumor pathogenesis and progression. A recent review by Alireza *et al*. summarizes numerous miRNAs discovered in glioma tissue and their function at the cellular level or in connection with tumor pathogenesis (5).

The LET-7 family, which includes *miR-LET-7a, miR-LET-7b* as well as 11 other members, was the first miRNA family found in the human genome (18, 19). Since then,

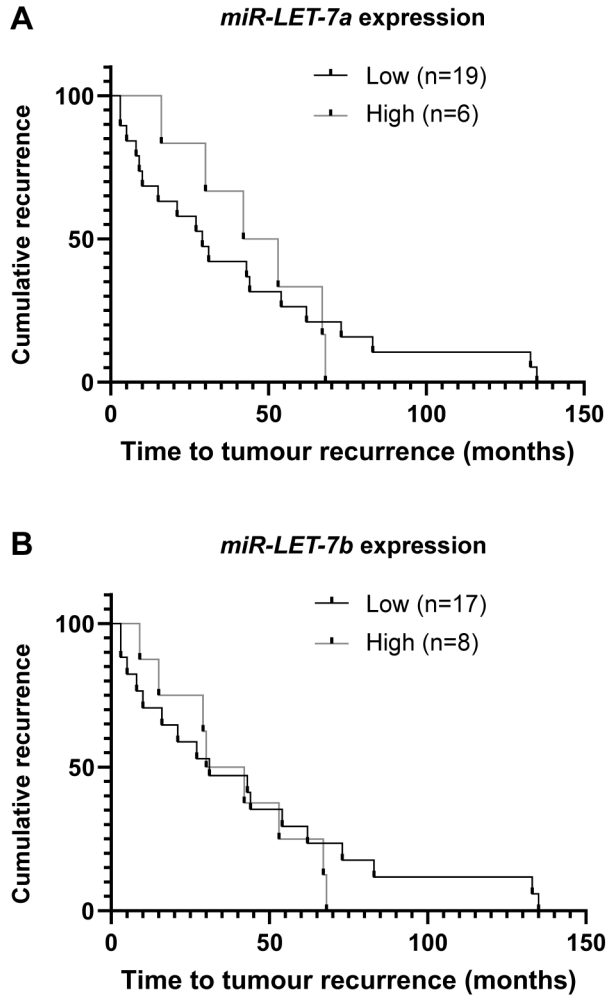

Figure 4. *Recurrence-free survival in patients according to miR- LET-7a (A) and miR- LET-7b (B) expression. (A) Using a cut-off of 1.42 for miR- LET-7a expression, the median times to recurrence for the groups with low and high expression were 29 versus 47.5 months, respectively (p=0.9). (B) Using a cut-off of 0.83 for miR- LET-7b expression, the median times to recurrence for the groups with low and high expression were 36 versus 31 months, respectively (p=0.6).*

this family has been identified as both oncogenic and tumor-suppressive in different cancer types (20). With respect to gliomas, *miR-LET-7a* and *miR-LET-7b* have been predominantly described as tumor suppressors (12-14).

It has been shown that an increase in expression of both miRNAs reduces tumor growth and malignant behavior of glioma cells, and that a lower expression is associated with a poorer prognosis (12-15). Thus, previous studies have demonstrated lower expression in patients with prognostically less favorable high-grade tumors compared to those with low-grade tumors (12, 15). In contrast, the attempt to establish a signature that can indicate recurrence in the longitudinal course of a patient has hardly been undertaken. To the best of our knowledge, there are only two studies to date that have detected a significant change in expression of various miRNAs in the first glioblastoma recurrence compared with the primary tumor, including *miR-LET-7a* and *miR-LET-7b* (16, 21).

The aim of our study was to extend the expression analysis of *miR-LET-7a* and *miR-LET-7b* up to the fifth relapse and to analyze all glioma classes to verify a universal relapse signature.

Longitudinal expression of miR-LET-7a and *miR-LET-7b* and *comparison of recurrence-free survival*. No consistent results were generated for the expression of these two miRNAs in the longitudinal course nor in the comparison of their expression by recurrence-free survival. In the longitudinal expression analysis, there were no other significant differences except for the down-regulation of *miR-LET-7a* and *miR-LET-7b* in the first recurrence and the primary tumor. However, it is evident for both miRNAs that all recurrences tended to have lower expression compared to the primary tumor. In addition, there was a tendency for decreasing expression up to the fifth recurrence. This, however, needs to be verified with a higher number of patients, as the number of samples was low, especially for the fourth and fifth relapses (n=5 and n=1, respectively).

Moreover, as shown by the Kaplan–Meier curve, only a tendency towards longer recurrence-free survival was detected with higher *miR-LET-7a* expression. Studies with a larger patient number may clarify this notable correlation.

Significant down-regulation of miR-LET-7a and *miR-LET-7b in the first relapse, independent of WHO tumor grade*. We found significant down-regulation of *miR-LET-7a* and *miR-LET-7b* in the first recurrence compared with the associated primary tumor. In addition, paired samples in which the WHO grade remained the same in the first relapse as in the primary were examined separately to exclude the possibility that the lower miRNA expression was only due to a higher WHO grade in the first relapse. This analysis showed consistent, significant down-regulation of both miRNAs in the first relapse.

Thus, to the best of our knowledge, we demonstrated for the first time that *miR-LET-7a* and *miR-LET-7b* are down-regulated not only in glioblastoma recurrences (16, 21), but also in all other glioma types and independently of the WHO tumor grade.

Correlation between expression level and *WHO tumor grade*. On the one hand, we were able to replicate the previously demonstrated finding of lower *miR-LET-7a* and *miR-LET-7b* expression with higher tumor malignancy (WHO III and IV) compared to low-grade tumors (WHO II) (12, 15). Secondly, we newly demonstrated significant down-regulation of both miRNAs not only from low- to high-grade tumors, but also within the high-grade group, *i.e.* between grades III and IV,

where, according to our study the strongest down-regulation of *miR-LET-7a* and *miR-LET-7b* occurred. This result supports previous studies that showed up-regulation of these tumor-suppressive miRNAs, which not only led to reduced tumor growth but also reduced malignancy (12, 14). Conversely, this means decreasing expression of *miR-LET-7a* and *miR-LET-7b* with increasing grade may explain the worsened prognosis in patients with glioblastoma compared to those with other glioma classes (22, 23).

Future perspectives. Firstly, it would be useful to confirm the results generated here in a study with a larger number of patients as we only had 25 cases that met our criteria of at least two recurrences and, of these, only one patient had a fifth recurrence. Moreover, in a future longitudinal study with a larger cohort, it would be interesting to confirm or refute the trend for down-regulation of *miR-LET-7a* and *miR-LET-7b* shown here.

The next step would be to move towards clinical application and verify *miR-LET-7a* and *miR-LET-7b* in blood samples from patients with glioma of any WHO class as signatures of recurrence. Expression analysis of miRNAs in blood samples from patients with glioma has been successful in previous studies (24-26). For example, only recently Billur *et al*. demonstrated that *miRNA582-5p* and *miRNA-363* were up-regulated in glioblastoma samples compared with normal tissue (26).

Conclusion

miR-LET-7a and *miR-LET-7b* represent a potential signature of recurrence for gliomas of any WHO grade.

Conflicts of Interest

The Authors declare no potential conflicts of interest exist with respect to the research, authorship, or publication of this article

Authors' Contributions

SW: Experimental work, writing of manuscript; SK: reading and correcting of manuscript, experiments; RG: correcting of manuscript; MT: idea, concept, manuscript revision, supervision.

Funding

This study was supported by the Koeln Fortune Program/Faculty of Medicine, University of Cologne, Germany.

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Received May 2, 2024
Revised October 25, 2024
Accepted October 30, 2024