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Impact of MiRNA-181a2 on the Clinical Course of IDH1 Wild Type Glioblastoma

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Abstract: Background: Recently, miRNA-181a2 could be identified as a major regulator of IDH1 expression in fat tissue. The IDH1 gene, its mutation and expression have a major impact on overall survival in patients with glioblastoma. The presented study aimed to investigate the effect of miRNA-181a2 on IDH1 expression in glioblastoma and on the prognosis of patients suffering from, for example, a tumor. Methods: A total of 74 glioblastoma specimens were analyzed for the expression of miRNA-181a2, acquired as fold change, using qRT-PCR. IDH1 protein expression was estimated via mRNA quantification. Eight post mortal, non-glioma related brain tissue specimens served as the control group. The results were correlated with relevant demographic and clinical aspects of the cohort. A TCGA dataset was used as an independent reference. Results: MiRNA-181a2 was significantly downregulated in tumor samples compared to the control group ($p < 0.001$). In the glioblastoma cohort, 63/74 (85.1%) showed an IDH1 wild type, while 11/74 (14.9%) patients harbored an IDH 1 mutation. In patients with IDH1 wild type glioblastoma, low miRNA-181a2 expression correlated with a prolonged overall survival ($p = 0.019$), also verifiable in an independent TCGA dataset. This correlation could not be identified for patients with an IDH1 mutation. MiRNA-181a2 expression tended to correlate inversely with IDH1 protein expression ($p = 0.06$). Gross total resection of the tumor was an independent marker for a prolonged survival ($p = 0.03$). Conclusion: MiRNA-181a2 seems to be a promising prognostic marker of selective glioblastoma patients with IDH1 wild type characteristics. This effect may be mediated via direct regulation of IDH1 expression.

Keywords: microRNA; glioblastoma; prognosis; epigenetic; miR-181a2

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1. Introduction

Glioblastoma (GBM) is a primary malignant brain tumor with a five-year survival rate of less than 3% [1]. It is characterized by aggressive biological behavior and high proliferation rates [2]. Currently, the gold-standard treatment for GBM combines surgical gross total resection and concomitant radio-chemotherapy with temozolomide [3]. Relevant factors that turned out to have impacted individual prognosis are age at diagnosis, extent of resection, Karnofsky performance score and methylation status of the O^6-Methylguanine-DNA Methyltransferase (MGMT) promotor [4–6]. The most important molecular predictor for survival is the mutation status of IDH1 and IDH2 within the tumor tissue [7]. Some authors ascribe the effect of IDH1 (R132H) mutation ($IDH1_{mut}$) on the clinical course of glioma in general to the accumulation of 2-hydroxyglutarate in tumor cells [8]. This metabolite is only produced in tumor cells harboring the IDH1 mutation, but not in cases of IDH1 wild type ($IDH1_{wt}$) [9]. In glioblastoma, IDH1 mutation, and therefore the potential production of 2-hydroxyglutarate, may result in prolonged survival [7].

Micro-RNAs (miRNA) are 20–25 base pairs long, single-stranded, non-coding RNAs, which post-transcriptionally regulate gene expression. They do so via binding to a specific messenger RNA (mRNA) of a transcripted gene. A perfect match of miRNA and mRNA leads to the decay of the mRNA, whereas a sequential match is causing a blockade of translation. In either case, this results in an impaired expression of the specific protein [10]. Exceptional expression of specific miRNAs in GBM has been proposed as a prognostic marker for the clinical course of the disease [11].

The miRNA-181 family consists of six members, -181a1, -181a2, -181b1, -181b2, -181c and -181d [12]. All members of this miRNA family seem to be downregulated in glioblastoma tissue compared to non-glioma related brain specimens [13]. Some of these miRNAs have also been linked to glioma genesis, progression and the response to specific GBM therapy strategies [14–16]. Moreover, miRNA-181a1 expression and connected methylation pathways have been linked to prolonged survival in GBM patients [17].

Recently, miRNA-181a2 was identified to regulate IDH1 expression in fat tissue [18]. Therefore, and due to the known impact of IDH1 expression on the course of glioblastoma, the question arises whether miRNA-181a2 expression itself also has an impact on prognosis.

2. Results

The expression level of miRNA-181a2 was assessed as fold change (FC), in relation to miRNA standard RNU48, in all 74 tumor specimens. Subsequently, these findings were correlated with clinical data, as well as the results of the methylation analysis of MGMT and the mutation analysis of IDH1.

2.1. MiRNA-181a2 in the Whole Collective Compared Non Glioma Brain Tissue

The whole glioma collective (n = 74) was characterized by a FC of miRNA-181a2 of 0.031 ± 0.03, [0.002–0.241]. A specimen of non-glioma related brain tissue showed an expression of FC = 0.15 ± 0.05 [0.09–0.24]. MiRNA-181a2 was significantly downregulated in tumor samples compared to the control group ($p < 0.001$). This is highlighted in Figure 1. There was no correlation of miRNA-181a2 expression with overall survival (OS) ($p = 0.58$) and progression-free survival (PFS) ($p = 0.62$) regarding the whole collective of 74 patients. MiRNA-181a2 level was also not associated with age at diagnosis ($p = 0.48$).

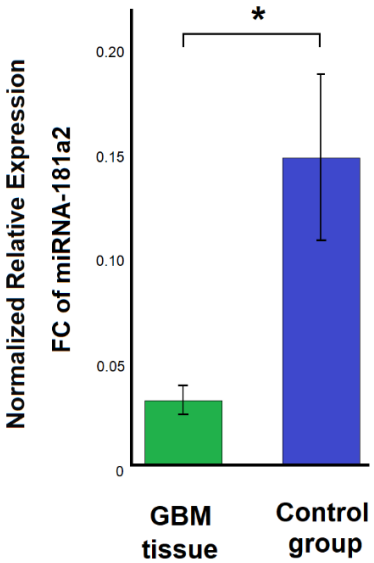

Figure 1. MiRNA-181a2 expression in GBM specimen (green, n = 74) and non-glioma related brain tissue of post mortal donors (blue, control group, n = 8) normalized to their respective RNU-48 expression. * indicates significance.

2.2. MiRNA-181a2 in Subgroup Analysis IDH1$_{wt}$ Tumors

A stratified analysis was performed for 63 patients with IDH1$_{wt}$ tumors. This subgroup showed an OS of 14.9 ± 15.6, [0.6–69.8] months. Progression-free survival was 9.3 ± 11.4, [0.2–58] months. Fold change of miRNA-181a2 expression was 0.026 ± 0.017, [0.002–0.07]. When applying a model of linear regression, an inverse correlation between expression of miRNA-181a2 and OS (p = 0.019) was observable. The same effect was seen regarding miRNA-181a2 expression and PFS (p = 0.021), as visualized in Figure 2.

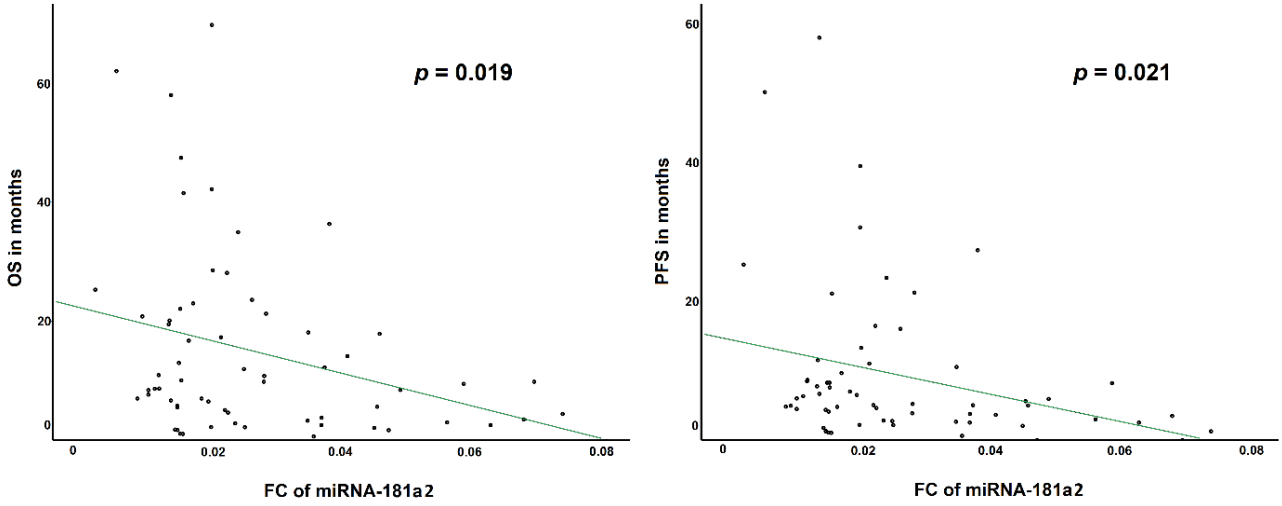

Figure 2. Linear regression model showing OS and PFS depending on miRNA-181a2 expression in IDH1$_{wt}$ tumors (n = 63). The X-axis represents the miRNA-181a2 expression expressed as fold change normalized to their respective RNU-48 expression. The left section highlights overall survival in dependence of miRNA-181a2 expression, the right section depicts progression-free survival in the dependence of miRNA-181a2 expression.

For clinical implementation, thresholds to distinguish the degree of expression are desirable. The IDH1$_{wt}$ (n = 63) collective was therefore dichotomized by referring to the mean expression of miRNA-181a2 in this group (FC = 0.026). There were 41/63 (65%) patients with a low expression of miRNA-181a2 (FC < 0.026). Overall survival and PFS were 18.1 ± 17.6, [1.1–69.8] months and 11.2 ± 12.9, [1.1–58] months, respectively. In this group, ten patients survived longer than 24 months. A high expression of miRNA-181a2 was found in 22/63 (35%) of the patients. OS within this group was 9.1 ± 8.5, [0.6–36.3] months. Progression-free survival of the 22 patients with a high miRNA-181a2 expression was 5.6 ± 6.6, [0.2–27.4] months. Only one patient in this group survived longer than 24 months. Log-rank-test showed a significant longer OS (p = 0.014) and PFS (p = 0.014) in patients with low miRNA-181a2 expression, as highlighted in Figure 3.

A TCGA dataset containing 32 patients with a IDH1$_{wt}$ glioblastoma and a therapy similar to the above mentioned 63 patients was obtained to validate these findings. A low miRNA-181a2 expression was associated with a prolonged survival (p= 0.02), as depicted in Figure 4.

2.3. MiRNA-181a2 in Subgroup Analysis IDH1$_{mut}$ Tumors

Data of 11 patients with an IDH1$_{mut}$ glioblastoma was further analyzed. This subgroup showed an OS of 33.2 ± 33.1, [5.7–76.2] months. Progression-free survival was 11.1 ± 10.1, [0.7–32.3] months. Fold change of miRNA-181a2 expression was 0.057 ± 0.06, [0.009–0.24]. There was no association between OS (p = 0.73) or PFS (p = 0.23) and the expression of miRNA-181a2.

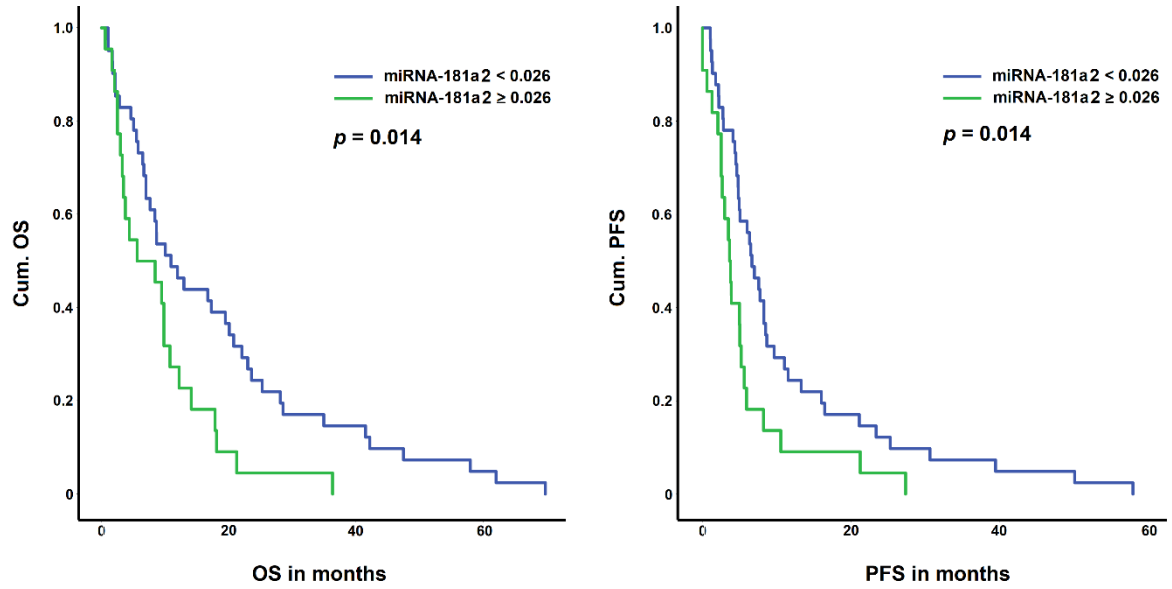

Figure 3. Kaplan-Meier curve of survival data for $IDH1_{wt}$ tumors. Overall survival (section A) and progression-free survival (section B) are shown for high (fold change FC $\geq$ 0.026, green curve) and low (FC < 0.026, blue curve) expression of miRNA-181a2. The X-axis represents time after diagnosis, and the Y-axis represents cumulative OS and PFS as appropriate. Cum OS = cumulative overall survival; Cum PFS = cumulative progression-free survival.

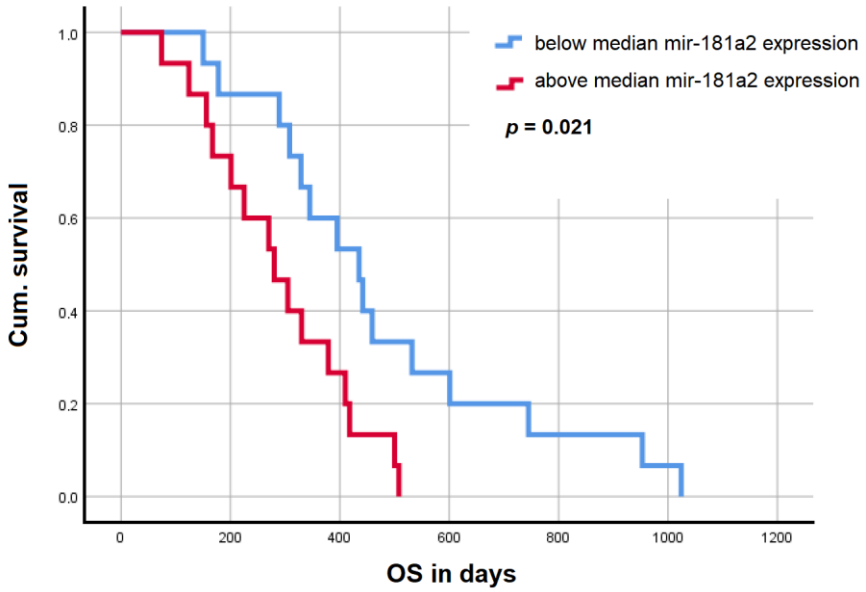

Figure 4. Kaplan-Meier curve showing the survival analysis of the TGCA dataset of 32 patients with $IDH1_{wt}$ GBM. The red and blue curves represent patients above and below the median miRNA-181a2 expression, respectively.

2.4. MiRNA-181a2 Binding Site on mRNA of IDH1

To identify interactions of miRNA-181a2 and the mature mRNA sequence of IDH1, a binding site analysis, via GenBank (https://www.ncbi.nlm.nih.gov/genbank/, accessed on 1 March 2021), was performed. It revealed binding sites at nucleotide positions 449, 451–457, 460 and 468 in the mRNA sequence of IDH1. The binding configuration is of an incomplete character. The results are shown in Figure 5.

Figure 5. Binding site of miRNA-181a2 on mature IDH1$_{wt}$ sequence. Complementary nucleotides are marked in red.

2.5. Effect of miRNA-181a2 Expression on IDH1 Expression

The impact of miRNA-181a2 on the prognosis of IDH$_{wt}$ tumors was evaluated by IDH1 protein expression analysis. Indirect measurement via mRNA expression of IDH1 was used. Of all the 63 IDH$_{wt}$ tumors, 51 were analyzed for protein expression (81%). Twelve cases had to be excluded of further analysis due to insufficient remaining tumor tissue. Fold change of mRNA of IDH1 was 0.234 ± 0.309, [0.0002–1.12]. Applying a model of linear regression, a trend ($p = 0.06$, $R = -0.55$) for an inverse correlation between the expression of miRNA-181a2 and mRNA expression of IDH1 could be identified, as highlighted in Figure 6. A high IDH1 protein expression seems to be linked to a low miRNA-181a2 expression.

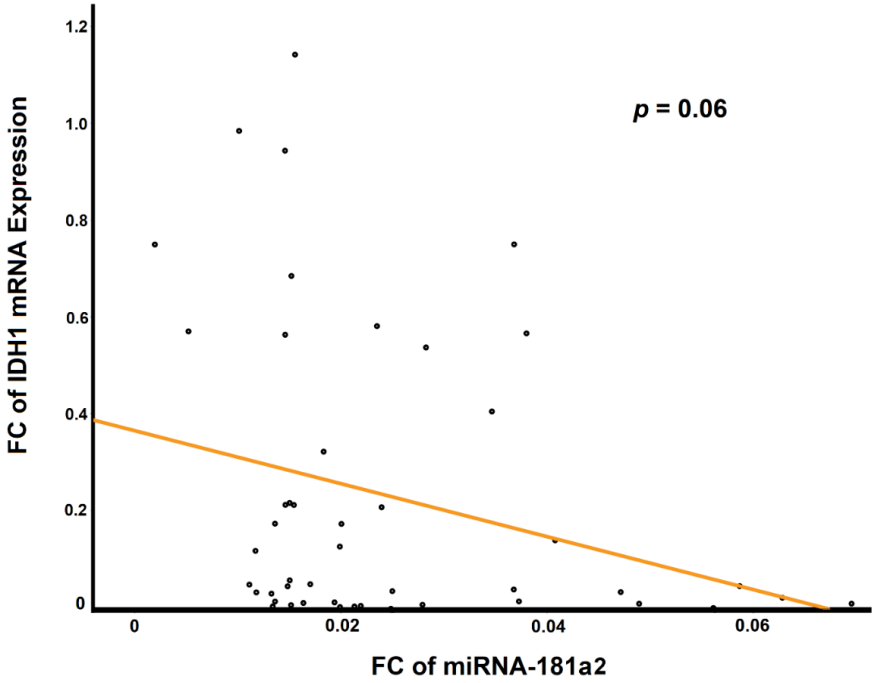

Figure 6. Linear regression model showing IDH1 mRNA expression as an estimation of IDH1 protein level, as a function of miRNA-181a2 expression in IDH1$_{wt}$ tumours. The X-axis represents the miRNA-181a2 expression expressed as fold change; the Y-axis represents IDH1 mRNA expression is also as a fold change.

2.6. Correlation with Further Clinical and Molecular Data in the Total Collective

IDH1 mutation correlated with a prolonged OS ($p = 0.004$) independently of miRNA-181a2. Patients with an IDH1$_{mut}$ tumor survived 33.25 ± 33.12, [5.65–76.2] months. In cases of an IDH$_{WT}$ tumor OS was 14.96 ± 15.59, [0.62–69.78] months.

MGMT promotor methylation was found in 37 (50%) of all cases. The residual 37 cases (50%) showed no methylation. Patients harboring a MGMT methylation survived 20.76 ± 26.26, [0.62–76.2] months. Those without an MGMT methylation survived 13.33 ± 12.55, [1.71–62.03] months. This difference was statistically significant ($p = 0.04$).

The extent of resection was determined by postoperative magnetic resonance imaging (MRI) within 48 h after surgery. A gross total resection (GTR) was defined as a lack of

detectable tumor remnants. GTR was possible in 24/74 (33.8%) of the cases. If any residual tumor was detectable, it was considered to be subtotal resection (STR). STR was identified in 44/74 (59.5%). In 5/74 cases (6.7%), determination was not possible due to missing postoperative MRI data. GTR was linked with prolonged overall survival ($p = 0.03$). Age at diagnosis was an independent predictor of survival. Patients of higher age showed a significantly decreased OS ($p = 0.001$).

2.7. Multivariate Analysis in the $IDH1_{wt}$ Cohort

The impact of miRNA-181a2 expression on survival in the $IDH1_{wt}$ cohort was eventually analyzed with respect to all the above-mentioned factors influencing survival. The detailed results of a model of multivariate linear regression for OS are highlighted in Table 1. MiRNA-181a2, age at diagnose, extend of resection as well as Karnofsky performance score and MGMT methylation were significant and independent predictors for prolonged survival within the study population. MiRNA-181a2 expression was not associated with any of the aforementioned clinical and molecular characteristics.

Table 1. Multivariate linear regression for OS in $IDH1_{wt}$ tumors.

Item	Regression Coefficient	Standard Error	HR	Sig.	HR 95% Lower	Range CI Upper
FC miRNA-181a2	−256.4	108.1	−2.37	0.021 *	−472.7	−40.1
Extend of resection	−0.95	0.97	−0.97	0.034 *	−0.91	1.01
Age at diagnosis	−0.48	0.173	−2.79	0.007 *	−0.83	−0.14
MGMT	−0.38	0.267	0.404	0.043 *	0.68	1.15
Karnofsky	−0.33	0.009	0.97	0.001 *	0.95	0.96

* indicates significance.

3. Discussion

Members of the miRNA-181 family have been shown to impact glioma genesis, progression and the response to specific GBM therapy strategies [14–17]. MiRNA-181a1 overexpression was shown to sensitize glioma cells to radiation treatment due to the downregulation of Bcl-2 expression [19]. Consecutively, miRNA-181a1 overexpression was suggested as a positive prognostic marker in GBM [17]. MiRNA-181b1 and -181b2 have been reported to function as tumor suppressors triggering growth inhibition, inducing apoptosis and inhibiting invasion in glioma cells [20].

All members of this miRNA family seem to be downregulated in glioblastoma tissue, as compared to a non-glioma related brain specimen [13]. This stands in concordance with our findings as GBM specimen in the study at hand displayed a significant downregulation of miRNA-181a2 compared to non-glioma brain tissue.

Irrespective of glioma specimen, literature refers to miRNA-181a2 as a regulator of IDH1 expression in non-neoplastic tissue [18]. The impact of IDH1 itself on survival in GBM is undisputed [21]. Main question of the study at hand was therefore whether miRNA-181a2 impacts IDH1 expression in GBM and whether miRNA-181a2 expression influences the clinical course of the disease, respectively.

In the study at hand, patients with $IDH1_{wt}$ GBM and a low miRNA-181a2 expression showed a significant prolonged overall survival compared to patients with $IDH1_{wt}$ tumors and a high miRNA-181a2 expression, also verifiable in an independent TCGA cohort. This effect of miRNA-181a2 demonstrated to be independent of other factors influencing survival in GBM in multivariate analysis. Interestingly no such effect was noticed within the group of patients suffering from an $IDH1_{mut}$ GBM. The authors are aware of a certain limitation resulting from the limited number of cases within the TCGA cohort with 32 patients and the IDH_{mut} sub-cohort with only 11 individuals. Since a reliable correlation is based on highly specific parameters, as mentioned above, potential reference cases of the TCGA database turned out to be as limited as presented.

The impact of IDH1 mutation on GBM prognosis is commonly attributed to the accumulation of an onco-neo-metabolite in $IDH1_{mut}$ cases. IDH1, soluted in the cytoplasm, catalyzes the oxidative decarboxylation of isocitrate for α-ketoglutarate in the citric acid cycle [22]. It therefore plays a crucial role in the energy metabolism of the cell. All cells, including tumor cells, require a functional IDH1 for survival and reproduction [23]. A mutation (R132H) of IDH1 in glioma cells prevents catalyzation of isocitrate to α-ketoglutarate. Instead $IDH1_{mut}$ catalyzes isocitrate to 2-hydoxyglutarate (2HG). This new metabolite cannot be further processed in the citrate cycle and therefore accumulates in GBM cells causing multiple epigenetic transitions, resulting in a less aggressive or invasive tumor compared to its IDH wildtype counterpart [8].

While the effect of miRNA-181a2 on prognosis in our study was only observed in IDH_{wt} tumors, 2HG expression may not have contributed to this effect as 2HG production is strictly bound to the IDH1 R132H mutation. This may be a hint that other IDH1 related mechanisms next to 2HG production in $IDH1_{mut}$ GBM cases may play a role in the progression of the disease. Hence the question arose, if IDH1 expression, independent of the R132H mutation and its consecutive accumulation of 2HG, may be regulated by miRNA-181a2.

The study at hand indicates a trend, that miRNA-181a2 inversely correlates with IDH1 expression in GBM. The authors are aware that the method used, measuring the IDH1 protein expression via its mRNA expression, has its limitations due to its indirect nature. Results may be biased due to a post-transcriptional mRNA modification, leading to an over- or underestimation of the corresponding IDH1 protein expression [24]. The reason for using this method despite its disadvantages was the lack of sufficient tumor material for further proteomic analysis. Hence, RNA isolates, initially used for miRNA expression acquisition, were used for protein expression analysis. In respect to the above-mentioned drawbacks, the presented results of the miRNA-181a2 impact on IDH1 protein expression should be scrutinized critically. Nevertheless, they are in line with the literature, as demonstrated in fat tissue [18].

After all, the data in the presented study offers a new perspective regarding IDH1 impact on GBM. Not only the catalyzation of 2HG in $IDH1_{mut}$ GBM cases may influence prognosis, but also miRNA-181a2 expression in their $IDH1_{wt}$ counterparts, regulating the very expression of IDH1 itself.

Further factors associated with OS and PFS were the age at diagnosis and the extent of tumor resection. In the study at hand, older patients had a significantly decreased OS. This aligns with other authors who reported a significantly reduced survival in older glioblastoma patients [25,26].

In the present study, patients undergoing GTR had a significantly longer OS, compared to those who only had STR. The positive prognostic effect of GTR was also independent of miRNA-181a2 expression in multivariate analysis. This finding, which is supported by multiple reports in the literature, underlines the tremendous importance of radical surgery in GBM treatment [27,28].

A limitation of the study is its retrospective design and the limited number of patients included especially regarding analysis of IDH1 protein expression. This is of importance regarding the considerable spread in the expression levels of the analyzed RNAs. However, these findings may be ascribable to the very nature of GBM disease. As the heterogeneity of this tumor entity is well known, widespread expression levels of district miRNAs may be a facsimile of this characteristic on an epigenetic level.

In conclusion, a low miRNA-181a2 expression seems to ameliorate prognosis in GBM $IDH1_{wt}$ patients—at least partly—via IDH1 regulation. In addition, this could enable a new therapeutic approach as, at least theoretically, miRNA-181a2 expression in tumors can be downregulated via an anti-miRNA-181a2 drug.

4. Methods

4.1. Patients

Inclusion criteria were defined as neuropathologically approved diagnosis of primary GBM, as well as the availability of sufficient tumor tissue for further analysis. The probes collected were graded by the current WHO classification of brain tumors. Specimens of 74 cases where included. Informed consent was given by all participants of the study. All patients underwent surgery with consecutive concomitant radio-chemotherapy (Stupp-Scheme) in our center between 2014 and 2018 with clinical follow-up available until 2020. Clinical data regarding the cohort is given in Table 2.

Table 2. Patient baseline characteristics and treatment details (SD = standard deviation).

	All Patients, (*n* = 74)
Mean age ± SD, [range] in years	59.1 ± 11.4, [35.7–83.2]
Karnofsky performance score, median	80
Gender, No, (%)	
Male	47, (63.5)
Female	27, (36.5)
MGMT status, No, (%)	
methylated	35, (47.3)
unmethylated	39, (52.7)
IDH1 status, No, (%)	
R132H mutation	11, (14.9)
Wild type	63, (85.1)
Extent of resection, No, (%)	
Gross total resection	25, (33.8)
Subtotal resection	44, (59.5)
Not available	5, (6.8)
Overall survival ± SD, [range] in month	17.7 ± 19.9, [0.6–76.2]
Progression-free survival ± SD, [range] in month	9.5 ± 11.2, [0.2–58]
Death by end of trial, No, (%)	72, (97.3)

Eight brain tissue probes deriving from patients who faded away not related with glioma were provided by the anatomical institute of Saarland University (control group). Informed and written consent for postmortal scientific use of the tissue was obtained from all donors. All specimens were collected from the primary motoric cortex within less than 12 h after death as recommended by literature [29–31]. All tumor specimens were snap frozen after removal and stored at −80 °C prior to further analysis. This study was approved by the local ethics commission (93/16).

4.2. MiRNA Analysis

Isolation of miRNA from tumor specimens was performed using a miRNeasy miRNA Isolation kit (Qiagen, Venlo, The Netherlands) according to the manufacturer's instructions. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was performed using an ABI 7900 real-time PCR system (Applied Biosystems, Waltham, MA, USA). Primers for miRNA-181a2 for TaqMan miRNA assays were purchased from Applied Biosystems (TaqMan MicroRNA Assay, ID 002317, Applied Biosystems, Waltham, MA, USA). A Transcription Kit for reverse transcription (Applied Biosystems, Waltham, MA, USA) and TaqMan Gene Expression Master Mix for quantitative real-time PCR (Applied Biosystems, Waltham, MA, USA) were used according to the manufacturer's protocol. PCR was conducted in triplicate including negative controls. Quantitative miRNA expression

data were calculated using the comparative CT method with RNU48 as an accredited and stable reference miRNA [32]. For miRNA-181a2, fold change (FC) was calculated as follows: $FC = 2^{-(\text{CT-miRNA-181a2} - \text{CT-RNU48})}$. The expression of miRNA-181a2 was normalized to RNU48. A miRNA-181a2 expression simmilar to RNU48 expression within the same specimen is rated 1.

4.3. IDH1 Protein Expression Analysis

Messenger-RNA (mRNA) expression of IDH1 was used as an indirect method to estimate the protein expression of IDH1. For these analyses, the RNA specimens acquired by miRNA isolation were used. Again, quantitative reverse transcription PCR was performed to analyze the expression values (TaqMan Gene Expression Assay, IDH1: ID Hs01855675_s1, and β-actin: ID Hs99999903). Reverse transcription was performed with random primers (High-Capacity cDNA Reverse Transcription Kit, Applied Biosystems). Quantitative mRNA expression data was calculated with β-actin as reference RNA. FC was calculated, as described before [11].

4.4. Methylation Analysis and IDH1-R123H Staining

MGMT was determined by methylation-specific polymerase chain reaction (MS-PCR), as described previously [33]. Immunohistochemistry for IDH1 mutation was conducted, as described previously [11].

4.5. TCGA Data

To obtain data for an independent cohort, miRNA-181a2 expression data from TCGA was downloaded via the xenabrowser platform (https://xenabrowser.net/, accessed 1 March 2021). In these experiments, miRNA expression was measured with microarrays (Agilent Human miRNA_8 x 15K). The dimensionless fluorescence signal captured on the microarrays was normalized to that of the TCGA project (so-called level 3 data) to facilitate relative comparisons of expression levels between specimen. Survival data was obtained via the same platform. In total, TCGA contained 671 GBM datasets. A subset of 32 patients meet the criteria of primary GBM, available miRNA-181a2 expression data, a $IDH1_{wt}$, as well as a documented therapy protocol. All patients in this subset received a concomitant radio-chemotherapy with temozolomide. Kaplan–Meier survival analysis was performed by stratifying the expression values of these 32 patients by the median miRNA-181a2 expression into high (≥median, 16 patients) and low (<median, 16 patients) expression categories.

4.6. Statistical Analysis

All statistical analyses were performed using SPSS v.23 (IBM, Armonk, NY, USA). A model of linear regression was used to analyze the correlation between miRNA expression and clinical parameters such as overall survival, progression-free survival, and age at disease onset. Progression was defined either radiologically as a new contrast dye enhancing tumor formation or death of the patient. Cox regression was used to analyze overall survival and progression-free survival. Potentially interfering clinical characteristics like the extent of resection, age at diagnosis and Karnofsky performance sore were considered for final analysis. A value of $p < 0.05$ was considered statistically significant. A value of $p < 0.10$ was considered to be a statistical trend. Standard deviation was presented by ± [15].

Author Contributions: C.S. wrote the paper, designed the study and performed miRNA as well as methylation analysis and is the corresponding author. L.S. performed miRNA, mRNA as well as methylation analysis. F.T. performed miRNA analysis and wrote sections of the paper. W.S.-S. is the neuropathologist who examined the tumor specimen. S.U. wrote sections of the paper. R.K. designed the study, organized the financing and wrote sections of the paper. J.O. is the head of the department, designed the study and supervised the trial. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: All authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers' bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements), or non-financial interest (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.

Ethical Approval: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Ethics Committee of General Medical Council of the State Saarland (NO 93/16, 28 June 2016).

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