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ORIGINAL ARTICLE - TUMOR - GLIOMA

miRNA-26a expression influences the therapy response to carmustine wafer implantation in patients with glioblastoma multiforme

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Abstract

Background Glioblastoma multiforme is the most frequent malignant brain tumor in adults being marked with a very poor prognosis. Therapy concept implies concomitant radio-chemotherapy and facultative implantation of carmustine-eluted wafer. Current literature suggests microRNA 26a expression in glioblastoma to interact with alkylating chemotherapy. Subsequently, the aim of this study was to investigate the correlation of miRNA-26a expression and carmustine wafer implantation and its potential usefulness as a predictive marker for therapy response.

Methods In total, 229 patients with glioblastoma multiforme were included into the final analysis. Of them, 80 cases were recruited from the Saarland University Medical Center for a retrospective matched-pair analysis stratified after therapy regime: One group (carmustine wafer group; $n = 40$) received concomitant radio-chemotherapy with carmustine wafer implantation. The other group (control group; $n = 40$) only received concomitant radio-chemotherapy. The results were confirmed by comparing them with an independent dataset of 149 patients from the TCGA database. All tumor specimens were evaluated for miRNA-26a expression, *MGMT* promoter methylation, and *IDH1* R132H mutation status, and the results were correlated with the clinical data.

Results Twenty-three patients in the carmustine wafer group showed low expression of miRNA-26a, while 17 patients showed a high expression. In the control group, 28 patients showed low expression, while 12 patients showed a high expression. The patients with high miRNA-26a expression in the carmustine wafer group were characterized by a significantly longer overall (hazard ratio [HR] 2.750 [95% CI 1.352–5.593]; $p = 0.004$) and progression-free survival (HR 3.091 [95% CI 1.436–6.657]; $p = 0.003$) than patients with low miRNA-26a expression. The 17 patients in the carmustine wafer group with high miRNA-26a expression showed a significantly longer progression-free survival ($p = 0.013$) and overall survival ($p = 0.007$) compared with the control group. There were no such correlations identified within the control group. TCGA datasets supported these findings.

Conclusions MiRNA-26a expression turned out to be a promising predictor of therapy response and clinical outcome in glioblastoma patients treated with carmustine wafer implantation. For evaluation of the role of miRNA-26a in a combined therapy setting, further studies are needed in order to translate general findings to the patient's individual situation.

Keywords miRNA-26a · Carmustine wafer · Epigenetic · Glioblastoma

This article is part of the Topical Collection on *Tumor - Glioma*

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Abbreviations

CI	Confidence interval
FC	Fold change
GBM	Glioblastoma multiforme
GTR	Gross total resection
HR	Hazard ratio
KPS	Karnofsky Performance Score
MGMT	O6-Methylguanine-DNA methyltransferase
miRNA	MicroRNA
MRI	Magnetic resonance imaging

MS-PCR	Methylation-specific polymerase chain reaction
OS	Overall survival
PFS	Progression-free survival
qRT-PCR	Quantitative reverse-transcription polymerase chain reaction
STR	Subtotal resection
TCGA	The Cancer Genome Atlas
TMZ	Temozolomide

Introduction

Glioblastoma multiforme (GBM) is a primary malignant brain tumor with a very poor prognosis [35]. In general, it is characterized by an aggressive biological behavior with infiltrative invasion and proliferation [31]. Age distribution among patients is all over the place with a peak incidence between 50 and 60 years [19, 43]. The age at diagnosis, extent of tumor resection, and the mutation status of *IDH1* are factors that show significant influence on the individual clinical course [21, 22, 39, 45]. GBM patients with *IDH1* R132H mutation live significantly longer than those with wild-type *IDH1* [21]. The specific interactions of IDH1 mutation leading to a prolonged survival are not fully understood, but they seem to be associated with extensive accumulation of 2-hydroxyglutarate metabolite [49]. .Standard therapy is based on a multimodal treatment regime consisting of surgery and concomitant radio-chemotherapy (Stupp-scheme) [44]. Methylation status of the *MGMT* promoter can be used to predict therapy response to radio-chemotherapy: Patients with a methylated *MGMT* promoter tend to benefit more from radio-chemotherapy than those without methylation [22]. Additional therapy option is given by the implantation of carmustine-eluted wafers after tumor resection to locally boost chemotherapy effect within the brain tissue adjacent to the resection cavity [28]. However, the benefit of this adjuvant therapy is disputed. Initial trials reported a longer overall survival (OS) in case of carmustine wafer implantation [13]. Conversely, a recent study by Pallud et al. [36] could not determine a long-term benefit with respect to OS in a cohort of 354 patients, between those treated with Stupp-scheme and carmustine wafer implantation and those treated with Stupp-scheme only [36, 48]. .Furthermore, some adverse effects such as surgical wound infection and cerebral edema can occur attributed to carmustine wafers [46]. In this context, specific and reliable molecular markers would be of great benefit for the decision making considering carmustine wafer implantation.

MicroRNAs (miRNAs) are short, noncoding RNA molecules that silence gene expression via posttranscriptional modification by targeting specific mRNAs [2, 5]. Subsequently, these mRNAs are degraded and the coded protein will be not or less expressed. Abnormal miRNA expression has been linked with various malignancies, including gliomas [10, 24, 32, 37]. The author's previously published data about miRNA-181d which may impact the therapy response to carmustine wafer implantation as a single marker could not explain the effect of therapy response on GBM patient receiving carmustine wafer in total [41]. However, a recent trial revealed miRNA-26a to impact chemotherapy resistance when alkylating chemotherapy agents are used [15]. Therefore, miRNA-26a came to the fore.

Moreover, studies identified miRNA-26a as a crucial regulator of the RB1-E2F pathway and therefore the progression and prognosis of glioblastoma [47, 52]. Hyperphosphorylated RB1 releases the active E2F transcription factor, which subsequently initiates DNA replication in the S-phase [16]. This is also the critical part of the cell cycle were alkylating chemotherapeutical agents like carmustine interfere [34]. Mutations and deletions of *RB1* are among the most frequently observed genetic alterations in glial tumors and can cause the tumor to exhibit more aggressive biological behavior [3, 4, 17, 33]. The expression of *RB1* can be regulated by gene promoter methylation or different expression levels of miRNA-26a [30, 33].

Therefore, the current study focuses on evaluating miRNA-26a expression in GBM patients as another prognostic marker for recommending carmustine wafer therapy. The working hypothesis is that miRNA-26a expression may influence the therapeutic survival responses (OS and progression-free survival) to carmustine wafer implantation. This could be the key point in determining whether GBM patients can be recommended for carmustine wafer implantation in addition to standard Stupp-scheme and furthermore lead to an improved individual therapy for patients harboring GBM in the future. Additionally, *MGMT* and *RB1* promoter methylation and *IDH1* mutation status were determined and compared to the clinical outcome given by PFS and OS.

Methods

Patients

The presented retrospective trial included data of 229 patients with glioblastoma disease. From these, 80 patients underwent surgery at the Saarland University Medical Center in Homburg between 2005 and 2015; clinical data was available from 2005 to 2019. This cohort is identical with the previously published cohort of patients with updated clinical characteristics [41]. The study was approved by the local German ethical board (Ethikkommission der Ärztekammer des Saarlandes, Saarbrücken, Germany, General Medical Council of the State Saarland, NO 93/16). Inclusion criteria were set as

neuropathological diagnosis of GBM (WHO IV), a temozolomide therapy during Stupp-scheme, and a sufficient amount of tumor tissue (> 1 g) for further analysis. The 80 patients from the Saarland University Medical Center in Homburg were divided in two groups (matched-pair analysis) on the base of therapy regiment, resulting in 40 matched patients in each group, respectively. One group (carmustine wafer group) received a standard concomitant radio-chemotherapy (Stupp-scheme) with carmustine wafer (Gliadel®, Archimedes Pharma, Mannheim, Germany) implantation during tumor resection. The second group (control group) received a standard concomitant radio-chemotherapy (Stupp-scheme) with tumor resection. The match criteria were age at diagnosis, gender, and Karnofsky Performance Score (KPS). All tissue samples were snap-frozen after tumor removal and stored at − 80 °C for analysis. Clinical details such as PFS, OS, extent of resection, age at diagnosis, gender, and initial KPS of these 80 patients are presented in Table 1. The characteristics of the control group were validated by comparing them with an independent dataset of 149 patients selected from The Cancer Genome Atlas (TCGA) database [7]. Same criteria were applied to the TCGA for validation purposes. However, there were no data sets including carmustine-wafer implantation and information on miRNA-26a collectable.

miRNA analysis

Isolation of miRNA from the tumor specimens was performed using the miRneasy miRNA Isolation Kit (Qiagen, Venlo, Netherlands) according to the manufacturer's instructions. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed using an ABI StepOnePlus real-time PCR system (Thermo Fisher Scientific, Waltham, MA, USA). miRNA-26a primers for Taqman miRNA assays were purchased from Thermo Fisher Scientific (TaqMan MicroRNA Assay; miRNA-26a, ID 000404; Thermo Fisher Scientific, Waltham, MA, USA). The Transcription Kit for reverse transcription (Thermo Fisher Scientific, Waltham, MA, USA) and TaqMan Gene Expression Master Mix for quantitative real-time PCR (Thermo Fisher Scientific, Waltham, MA, USA) were used according to the manufacturer's instructions. PCR was performed in triplicate along with a negative control without a template. Quantitative miRNA expression data were estimated using the comparative CT method with RNU48 (TaqMan MicroRNA Assay, RNU48, ID 001006; Thermo Fisher Scientific, Waltham, MA, USA) as a proven stable reference miRNA [29]. Expression of miRNA-26a was normalized with that of RNU48. MiRNA fold-change (FC) was calculated using the equation, $FC = 2^{-(CT\text{-}miRNA\text{-}26a - CT\text{-}RNU48)}$.

Methylation analysis

DNA isolation was performed using a DNA isolation kit (QIAamp DNA Mini Kit 50; Qiagen, Venlo, Netherlands). Methylation status of the promoter regions of *MGMT* and *RB1* was determined using methylation-specific polymerase chain reaction (MS-PCR). DNA (500 ng) from each tumor specimen was bisulfite-treated (EZ DNA Methylation-Gold Kit 200; ZYMO RESEARCH, Irvine, CA, USA) [25]. Thus, unmethylated cytosine is converted to uracil, whereas methylated cytosine remains unchanged. The modified DNA was recovered by ethanol precipitation, and was suspended in PCR grade water. Primer sequences (Eurofins Genomics, Ebersberg, Germany) listed in Table 2 were used for analyzing the methylation status of *MGMT* and *RB1* [14, 40]. PCR products were electrophoretically separated using a 2% agarose gel. As a positive control, a globally methylated DNA was used (Bisulfite-Converted Human DNA Standard; ZYMO RESEACH, Irvine, CA, USA). Genomic DNA isolated from non-neoplastic dura mater tissue was used as a negative control. It is based on previously published research results where DNA from the dura mater showed reliable negative methylation at the promoters of MGMT and RBl [41, 42].

IDH1-R123H staining

Immunohistochemistry was performed using 5-μm-thick formalin-fixed, paraffin-embedded (FFPE) tissue sections mounted on StarFrost Advanced Adhesive slides (Engelbrecht, Edermünde, Germany), followed by drying at 80 °C for 15 min. Staining was performed on a BenchMark Ultra immunostainer (Ventana Medical Systems, Tucson, AZ, USA). The tissue sections were stained with anti-*IDH1*-R132H antibody H09 (Dianova, Hamburg, Germany) as previously published [8].

TCGA dataset

Control group data were validated with an independent validation group established using expression data from an Agilent Human miRNA_8x15k array, which was retrieved from the Gene Expression Omnibus platform (accession id GSE91014). These data sets were produced as part of the TCGA glioblastoma project [7]. TCGA level 3 data that is normalized to facilitate relative comparisons of expression levels across samples was downloaded. TCGAbiolinks R package was also used to map the expression values to clinical patient data from Genomics Data Commons (https://gdc.cancer.gov/) via the TCGAbiolinks R package [11]. Only a subset of 149 patients received treatment after the Stupp-scheme. For this subgroup, Kaplan–Meier survival analysis was performed in R. Kaplan–Meier plots were produced using the survminer R package. Validation for the carmustine wafer

Table 1 Details of the patients from the Saarland University Medical Center

	All patients ($n = 80$)	Carmustine wafer group ($n = 40$)	Control group ($n = 40$)	Statistical testing carmustine wafer group vs. control group
Treatment		Surgery with carmustine wafer implantation + concomitant radio-chemotherapy	Surgery + concomitant radio-chemotherapy	
Mean age ± SD [range], years	61.5 ± 10.7, [39.5–83.1]	60.9 ± 10.9, [39.5–80.1]	62.1 ± 10.6, [42.9–83.1]	$p = 0.91$
Median Karnofsky performance score	80	80	80	
Sex, no, (%)				
Male	54, (67.5)	27, (67.5)	27, (67.5)	$p = 1$
Female	26, (32.5)	13, (32.5)	13, (32.5)	
MGMT promoter status, no, (%)				
Methylated	36, (45)	16, (40)	20, (50)	$p = 0.37$
Unmethylated	44, (55)	24, (60)	20, (50)	
IDH1 status, no, (%)				
R132H mutation	3, (3.75)	2, (5)	1, (2.5)	$p = 0.56$
Wild type	77, (96.25)	38, (95)	39, (97.5)	
Extent of resection, no, (%)				
Gross total resection	26, (32.5)	15, (37.5)	11, (27.5)	$p = 0.3$
Subtotal resection	51, (63.75)	23, (57.5)	28, (70)	
Not available	3, (3.75)	2, (5)	1, (2.5)	
Mean overall survival ± SD [range], months	13.6 ± 13.1, [0.6–69.8]	14.9 ± 15.0, [1.0–69.8]	12.5 ± 10.5, [0.6–42.1]	$p = 0.29$
Mean progression-free survival ± SD [range], months	9.1 ± 9.5, [0.6–50.1]	10.3 ± 11.4, [1.0–50.1]	7.8 ± 7.1, [0.6–30.6]	$p = 0.28$
Deaths by end of trial, no, (%)	79, (98.8)	39, (97.5)	40, (100)	$p = 0.31$

SD standard deviation

Table 2 *MGMT* and *RB1* primer sequences for MS-PCR with annealing temperatures

MGMT	RB1
(methylated, 122 bp, 54 °C)	(methylated, 172 bp, 55 °C)
forward:	forward:
5`-GTTTTTAGAACGTTTTGCGTTTCGAC-3`	5`-GGGAGTTTCGCGGACGTGAC-3`
reverse:	reverse:
5`-CACCGTCCCGAAAAAAAACTCCG-3`	5`-ACGTCGAAACACGCCCCG-3`
(unmethylated, 129 bp, 56 °C)	(unmethylated, 172 bp, 55 °C)
forward:	forward:
5`-TGTGTTTTTAGAATGTTTTGTGTTTTGAT-3`	5`-GGGAGTTTTGTGGATGTGAT-3`
reverse:	reverse:
5`-CTACCACCATCCCAAAAAAAAACTCCA-3`	5`-ACATCAAAACACACCCCA-3´

bp base pair length of the resulting PCR product

group was not possible because no patients in the TCGA database could be found who received carmustine wafer implantation in combination with Stupp-scheme therapy.

Statistics

All statistical analyses were performed using SPSS version 23 (IBM, Armonk, USA). A linear regression model as well as Wilcoxon signed-rank test and chi-squared test were used to analyze the correlation between miRNA expression and clinical parameters such as OS, PFS, age at disease onset, and the frequency of wound-healing disorder. Kaplan–Meier analysis and Cox regression was used to analyze OS and PFS. Progression was defined either radiologically as a new contrast dye enhancing tumor formation in MRI imaging, death of the patient, or a reduction in KPS of over 30. A multivariant analysis was performed to evaluate the dependency of miRNA-26a expression of other acquired data like *MGMT* methylation status, *IDH1*-mutation, and extend of resection. Wilcoxon signed-rank test was used for comparing the extent of resection as well as comparison between the carmustine wafer group and control group. A p value of < 0.05 was considered statistically significant.

Results

The study's aim was to evaluate miRNA-26a as a molecular marker for predicting the therapy response to carmustine wafer implantation. Therefore, miRNA-26a expression was analyzed in all specimens ($n = 80$). The fold-change in miRNA-26a expression was 0.47 ± 0.26, ranging from 0.06 to 1.06, referring to mean normalized miRNA-26a expression. The patients were divided on the basis of a mean FC of 0.47 in those with high (FC ≥ 0.47) and low (FC < 0.47) miRNA-26a expression. Dichotomization of the data was used for better visualization via Kaplan–Meier curves. Analysis was also performed using continuous variables with Cox regression. By the end of the study, only one patient belonging to the carmustine wafer group was still alive, while the other 79 were dead. No *RB1* promoter methylation was found in any tumor specimens.

miRNA-26a expression in the carmustine wafer group

In the carmustine wafer group, OS correlated significantly with miRNA-26a expression when analyzed via linear regression ($p = 0.002$; Fig. 1a). A similar result was found regarding PFS as highlighted in Fig. 1b ($p = 0.001$). When analyzed as a continuous variable with Cox regression, high miRNA-26a expression was significantly associated with an ameliorated PFS (HR 3.091 [95% CI 1.436–6.657]; $p = 0.003$) and OS (HR 2.750 [95% CI 1.352–5.593]; $p = 0.004$). For visualization with Kaplan–Meier curves, the 40 patients of the carmustine wafer group were divided into 23 patients with low miRNA-26a expression and 17 with a high expression. The log-rank test showed a similar result to that of the Cox regression: PFS ($p = 0.003$) and OS ($p = 0.004$) were significantly longer for patients with high miRNA-26a expression (Fig. 2). The 23 patients in the carmustine wafer group with low miRNA-26a expression showed a PFS of 5.6 ± 4.0 months and an OS of 9.26 ± 7.77 months. The 17 patients in the carmustine wafer group with high miRNA-26a expression showed a PFS of 16.27 ± 15.26 months and an OS of 22.01 ± 19.45 months. In multivariant analysis, PFS and OS was significantly improved by miRNA-26a expression independent of MGMT-methylation status, IDH1-mutation status, extent of resection, and KPS.

miRNA-26a expression in the control group

In the control group, 28 patients showed low miRNA-26a expression, while 12 patients showed a high expression. The expression of miRNA-26a had no significant influence on the PFS (HR 1.170 [95% CI 0.402–1.629]; $p = 0.55$) or OS (HR 1.170 [95% CI 0.589–2.327]; $p = 0.657$) in the control group.

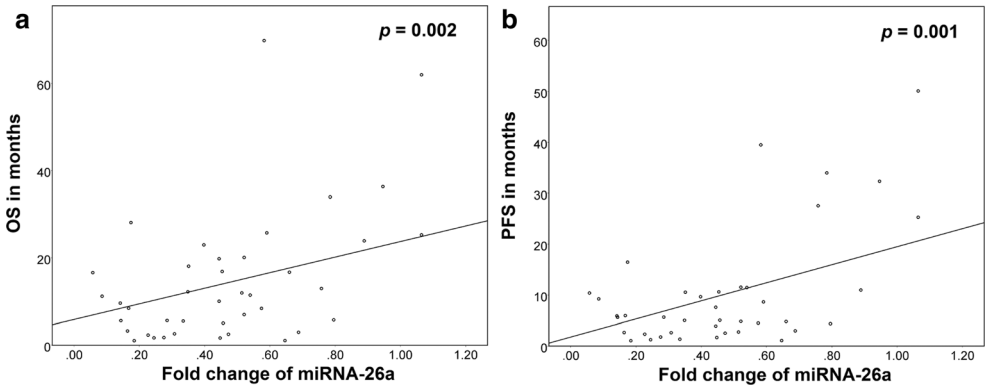

Fig. 1 Data on the carmustine wafer group. **a** Fold-change in miRNA-26a expression vs. OS. **b** Fold-change in miRNA-26a expression vs. PFS. X-axes represent time after diagnosis; Y-axes represent cumulative OS in **a** and PFS in **b**

A comparable result was found in the TCGA validation dataset. The 149 patients of the TCGA dataset were also divided according to the mean hsa-miR-26a expression value (≥ mean, 69 patients) and low (< mean, 80 patients). Figure 3 highlights the results of this validation cohort. There was no significant impact of miRNA-26a expression on OS in the TCGA dataset ($p = 0.57$).

Carmustine wafer group versus control group

There was no significant difference in miRNA-26a expression between the control and carmustine wafer groups ($p = 0.245$). There was also no significant difference between the carmustine wafer group and the control group with respect to OS ($p = 0.287$) and PFS ($p = 0.277$) when the Wilcoxon signed-rank test was used. Initial hypothesis was that miRNA-26a expression could predict subsets of patients that would benefit from carmustine wafer implantation. Overall survival and PFS characteristics of the 17 patients in the carmustine wafer group with high miRNA-26a expression were compared with those of the 12 patients of the control group who also showed high miRNA-26a expression. The OS was significantly prolonged for patients with additional carmustine wafer implantation (log-rank test; $p = 0.039$). Moreover, there was a trend toward a prolonged PFS in the carmustine wafer group (log-rank test; $p = 0.066$). PFS and OS of the 17 patients with high miRNA-26a expression in the carmustine wafer group were also compared with those of all the 40 patients of the control group. The 17 patients showed a significantly longer PFS (log-rank test; $p = 0.013$) and OS (log-rank test; $p = 0.007$) than that of the control group (Fig. 4). Again, the impact of miRNA-26a on PFS and OS was evaluated via multivariant analysis. The described results again were independent of MGMT-methylation status, IDH1-mutation status, extent of resection and KPS.

Multivariate analysis with previously published data of miRNA-181d

As previously published, miRNA-181d seems to impact therapy response to carmustine wafer [41]. Therefore, multivariate analysis regarding miRNA-181d, miRNA-26a, PFS, and OS was performed. The effect of miRNA-26a on PFS and OS in the carmustine wafer group was independent of miRNA-181d expression ($p = 0.34$).

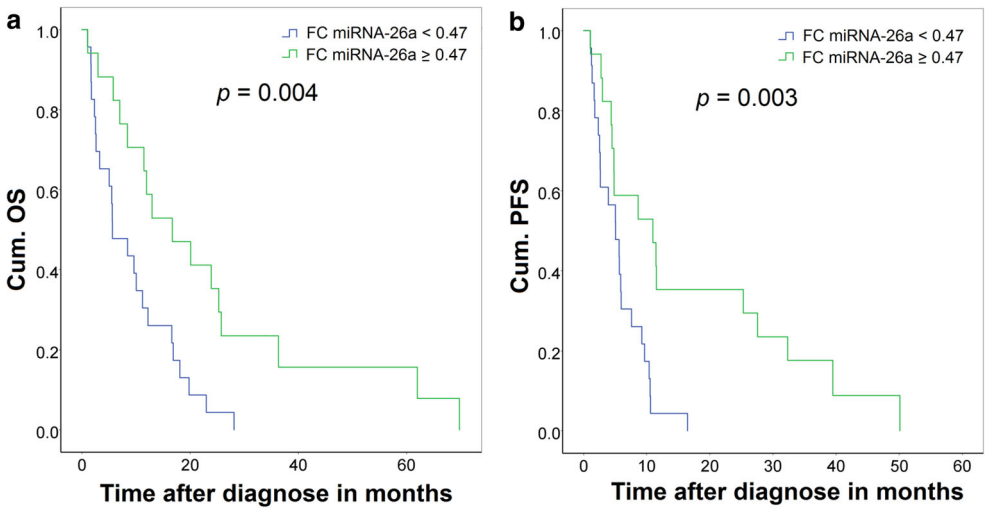

Fig. 2 Data on the carmustine wafer group. OS (**a**) and PFS (**b**) are shown in relation to high (green curve) or low (blue curve) expression of miRNA-26a. X-axes represent time after diagnosis; Y-axes represent cumulative OS in **a** and PFS in **b**. *Cum OS* cumulative overall survival, *Cum PFS* cumulative progression free survival

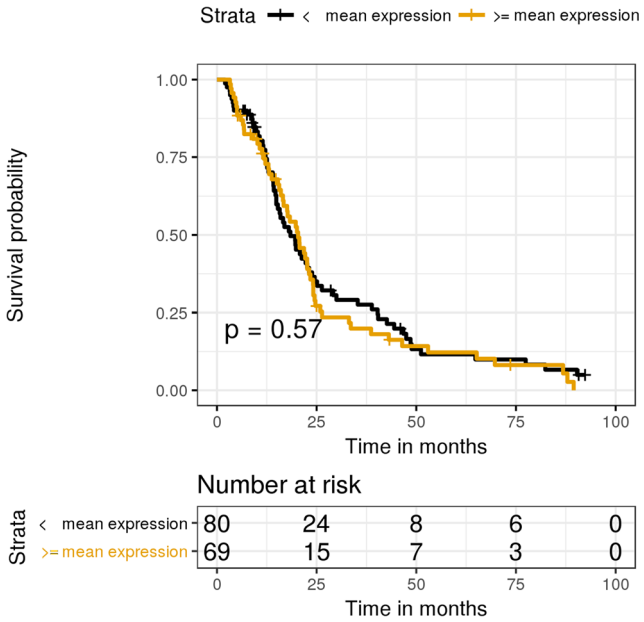

Fig. 3 Kaplan–Meier curve for the TCGA dataset, including 149 patients who only received the Stupp protocol therapy similar to the control group of the present study. X-axes represent the time after diagnosis; Y-axes represent OS

MGMT, IDH1, and clinical data

In the carmustine wafer group, 40% of the patients (16/40) harbored a methylated *MGMT* promoter. However, no influence on the OS ($p = 0.684$) or PFS ($p = 0.670$) was shown. In the control group, 50% of the patients (20/40) harbored a methylated *MGMT* promoter. Akin to the carmustine wafer group, the methylated *MGMT* promoter showed no impact on the OS ($p = 0.988$) or PFS ($p = 0.405$) in the control group. There was no correlation between *MGMT* promoter methylation status and miRNA-26a expression.

One patient in the control group and two patients in the carmustine wafer group harbored an *IDH1* R132H mutation. The *IDH1* R132H mutation showed no influence on the PFS or OS. Extent of resection was determined by postoperative MRI within 48 h after surgery. Gross total resection (GTR) was achieved if no tumor remnants were detectable. If any residual tumor was detectable, subtotal resection (STR) was ascribed. In 3 of the 80 patients (3.8%), determination of the extent of resection was not possible owing to the lack of a postoperative MRI; the details are listed in Table 1. The GTR had a significant impact on a long OS ($p = 0.004$) and PFS ($p = 0.023$). The patients with GTR showed an OS of 21.1 ± 20.1 months. The patients with STR showed an OS of 10.7 ± 8.9 months. There was no significant difference in distribution of extent of resection between carmustine wafer and control group ($p = 0.30$). In 12.5% (5/40) of the patients of the carmustine wafer group, a wound-healing disorder was found, which also occurred in 5% (2/40) of the patients belonging to the control group. The higher frequency in the carmustine wafer group did not reach a statistically significant threshold compared to that of the control group ($p = 0.235$). There was no correlation between the extent of resection or occurrence of wound-healing disorder and the expression of miRNA-26a. Age at diagnosis was an independent predictor of survival—older patients showed a significantly decreased OS (linear regression; $p = 0.008$).

Discussion

Without doubt, GBM diagnosis comes along with a devastating prognosis for the patient [20]. Gold standard therapy including a combined regiment of surgery and concomitant radio-chemotherapy provides an overall survival of just over 1 year [44]. The additional implantation of carmustine wafer with consecutive locally active chemotherapy is possible; however, this therapy comes along with several adverse effects and the benefit is disputed [36, 46, 48]. Molecular

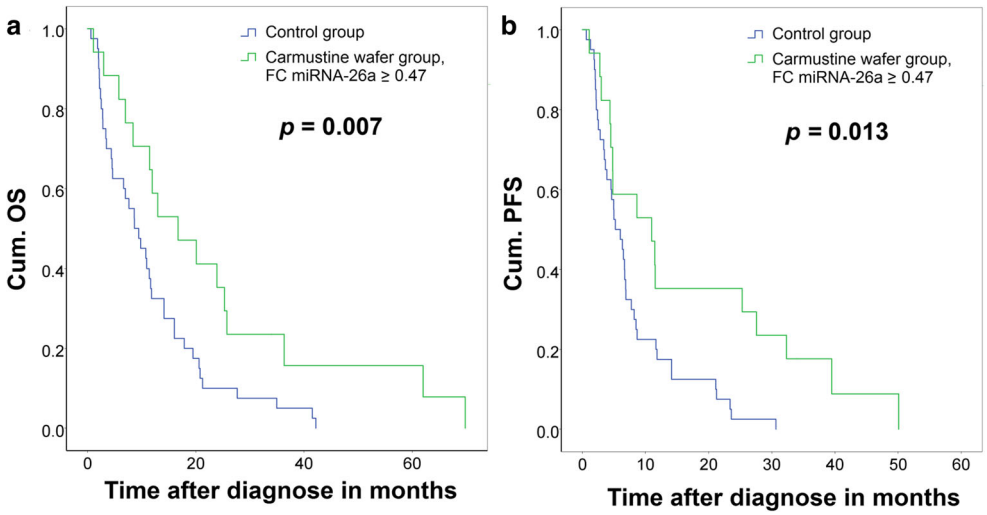

Fig. 4 Comparison between control group ($n = 40$, high and low miRNA-26a expression; blue curve) and the carmustine wafer group ($n = 17$, high miRNA-26a expression; green curve). X-axes represent time after diagnosis; Y-axes represent cumulative OS in **a** and PFS in **b**. *Cum OS* cumulative overall survival, *Cum PFS* cumulative progression free survival

markers that can be used in predicting a therapy response to carmustine wafer implantation would be beneficial. This could guide through the decision-making process whether carmustine wafers should be part of the treatment concept or not.

miRNA-26a expression affects therapy response in carmustine wafer but not in the control group

A significant correlation was found between a high miRNA-26a expression and prolonged PFS and OS in the carmustine wafer group. In contrast, miRNA-26a expression did not influence PFS or OS in the control group. The findings within the control group were supported by analysis of the independent TCGA validation cohort. A comparable validation was not possible for the carmustine wafer group because the TCGA database did not contain any patients who underwent both carmustine wafer implantation and Stupp protocol therapy.

MiRNA-26a has been identified previously as a regulator of *RB1* in esophageal adenocarcinoma cell culture [52]. Similar findings were presented in cases of colon cancer, where miRNA-26a regulated *RB1* [30]. An aberrant *RB1* is frequently found in glial tumors, and can produce aggressive tumors [3, 17]. In particular, a complete loss of *RB1* is associated with poor prognosis in the case of anaplastic astrocytoma [4]. In this context, the impact of miRNA-26a expression on therapy response to carmustine wafers can be explained via the regulation of RB1. RB1 protein is crucial for progression within the S-phase of cell cycle. Yet the S-phase is also the part of the cell cycle where alkylating chemotherapeutical agents such as carmustine interfere [34]. As the authors did not analyze the expression of RB1, which represents a certain limitation of the present study, our findings do not imply that the role of miRNA-26a expression in GBM relies exclusively on an *RB1*-based mechanism. Furthermore, miRNA-26a also plays a role in the regulation of PTEN [27, 51]. However, it remains difficult to explain why the impact of miRNA-26a expression on therapy response was only seen in patients belonging to the carmustine wafer group and not within the control group.

Patients of the carmustine wafer group with a high miRNA-26a expression show a better clinical course than patients of the control group

There was no difference in the PFS and OS between carmustine wafer and control groups. The OS of the carmustine wafer group was 14.7 ± 15.1 months, and that of the control group was 12.5 ± 10.5 months. The huge standard deviation results mainly from a few long-term survivor patients in both groups. Accordingly, other studies reported similar survival rates [26].

The benefit of carmustine wafer implantation is debatable. In 2003, Westphahl et al. performed a placebo-controlled phase III trial with 240 patients, which demonstrated a prolonged OS and PFS in patients who underwent carmustine wafer implantation [48]. However, the recent findings of Pallud et al. could not confirm this positive trend toward OS and PFS, thereby hampering the widespread use of carmustine wafers [36]. They performed a two-arm multicenter study with 354 patients. One arm received concomitant radio-chemotherapy, and the other arm received concomitant radio-chemotherapy with carmustine wafer implantation. It is therefore important to define subgroups of patients who may be able to benefit more from carmustine wafer implantation. The authors already reported miRNA-181d to impact therapy response to carmustine wafer implantation [41].

The initial prediction was that miRNA-26a expression directly influences PFS and OS. The patients belonging to the carmustine wafer group with high miRNA-26a expression showed a significantly longer PFS and OS than did the control group in total. For statistical precision, the patients of both groups with high miRNA-26a expression were studied. Patients of the carmustine wafer group with high miRNA-26a expression showed a significantly longer OS than did the patients of the control group with high miRNA-26a expression. In multivariant analysis, this effect of miRNA-26a expression on PFS and OS turned out to be independent from all other factors including patient characteristics as well as the expression of the previously reported miRNA-181d.

Further influence factors for OS and PFS, and limitations of the study

Influence factors for OS and PFS include the age at diagnosis and extent of tumor resection. In accordance with current literature, younger patients showed a significantly longer OS than older patients [12, 45] Moreover, patients with GTR showed a significantly longer PFS and OS than the patients with STR. This highlights the importance of radical surgery in patients with GBM and corresponds to previously published findings [1, 9, 18, 39].

In the present study, *IDH1* R132H mutation showed no influence on PFS or OS. However, in the literature, *IDH1* mutation status has been described as an important positive prognostic marker of GBM as patients harboring the *IDH1* R132H mutation usually show a significantly prolonged survival [21]. This apparent discrepancy is attributable to the low number of patients with *IDH1* R132H mutation ($n = 3$) included into this study. Due to the small amount of mutated IDH1 cases in the presented cohort, a conclusion about the impact of IDH1 mutation on therapy response to carmustine wafer should not be drawn.

Literature reports *MGMT* promoter methylation as a strong predictor of TMZ response [22, 23]. The data presented in this

trial does not support this. It must be noted that assessing *MGMT* promoter methylation status can be difficult [6]. A restriction of the MS-PCR method is given by the focus on islands determined by the sequence of the MS-PCR-primers, whereas potential methylation loci beyond that range are not encompassed. It is also recognized in the literature that different methods of determining *MGMT* methylation status, such as MS-PCR, semiquantitative MS-PCR, and single nucleotide resolution techniques via pyrosequencing, may produce divergent results [50]. The authors also note that recording *MGMT* methylation status as methylated or unmethylated, even routinely, may not reflect biological reality in which in-between states might exist.

Wound-healing disorder occurs in patients with carmustine wafer implantation at a frequency of 10–23% [38]. In the presented study, 12.5% of the patients were affected by it in the carmustine wafer group. However, there was no significant difference to the control group.

Conclusion

The results suggest that GBM patients with high miRNA-26a expression benefit more from combined carmustine wafer implantation and the standard radio-chemotherapy than from isolated radio-chemotherapy. However, further evaluation of miRNA-26a expression in a prospective trial as a predictive marker for therapy response to carmustine wafer implantation is recommended. The presented data also has to be scrutinized critically due to its single center character and the total number of 80 patients.

MiRNA-26a expression was measured retrospectively using tumor specimens acquired during the surgery. It would be beneficial to assess the expression of miRNA-26a in the tumor prior to surgery in order to help the surgeons decide preoperatively whether carmustine wafer should be implanted or not. Further prospective studies should investigate if these biomarkers are detectable reliably in blood or cerebrospinal fluid in order to identify suitable patients for carmustine wafer implantation prior to surgery. The present results underline the importance of stratifying patients on the basis of molecular markers in order to define a personalized therapy regiment and optimize the individual clinical outcome.

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Availability of data The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Compliance with ethical standards

All procedures performed in this study were in accordance with the ethical standards of the 1964 Helsinki declaration. This article does not contain any studies with animals performed by any of the authors.

Ethics approval and consent to participate This study was approved by the local German ethical board (Ethikkommission der Ärztekammer des Saarlandes, Saarbrücken, Germany).

Consent for publication Written informed consent was obtained from all patients (General Medical Council of the State of Saarland, NO 93/16).

Competing interests The authors declare that they have no competing interests.

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