

# Comparing tumor microRNA profiles of patients with long- and short-term-surviving glioblastoma

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**Abstract.** Glioblastoma is one of the most frequent primary brain tumors with a poor prognosis. Nevertheless, some patients show a prolonged survival. The aim of the present study was to compare the expression profiles of tumor derived microRNA (miR) of long-term survivors with those of short-term survivors in order to identify differentially expressed miRs as well as their target genes, which may elucidate mechanisms that play a role in varying tumor progression and, therefore, may influence survival. Formalin-fixed paraffin-embedded samples of 23 patients with glioblastoma were classified according to overall survival. Profiles of miR expression were determined using Nanostring technology. Expression levels of potential target genes of differentially expressed miRs were assessed using immunohistochemistry. MiR profiles of long-term survivors differed from those of short-term survivors. A total of three prominent differentially expressed miRs were highlighted: MiR-130b-3p, which is downregulated in long-term survivors, and miR-146b-5p and miR-148a-3p, which are upregulated in long-term survivors. Known tumor suppressor genes are among targets potentially affected by miR-130b-3p, whereas targets of miR-146b-5p and miR-148a-3p consist of several genes known to have a role in tumor invasion and aggressiveness. In conclusion, it was revealed that a type of miR-signature was associated with short- and long-term survival, potentially serving as biomarker for disease progression and providing a base for further functional studies.

## Introduction

Glioblastoma (GBM) is the most frequent and fatal primary brain tumor. The common therapy regimen consisting of surgery, radiotherapy, and chemotherapy with Temozolomid

(TMZ) (1,2) lack long lasting effects. The prognosis is dismal with a 5-year survival probability of 6.8% (varying with age at diagnosis) (3,4) which barely improved for decades (5), and a median overall survival (OS) of about 15-18 months after diagnosis which still remains at this low level despite several efforts (1-4).

Diverse therapeutic options are subject of actual or recent clinical studies, among them targeted approaches consisting of small-molecule kinase inhibitors (6-8) or antibodies (7), immunotherapy (9) or oncolytic viruses (10). But on the whole, the effects on OS remain rather low. Reasons for therapy failure include development of resistances caused by genetic heterogeneity or redundant signaling cascades, toxicity and other undesired side-effects, and low abundant drug availability on the tumor site due to inhibition by the blood-brain-barrier (6,7).

Nevertheless, some patients show a prolonged OS up to several years.

To date, several different factors, clinical and molecular, are discussed to cause or correlate with long-term survival (LTS). The possibility of gross tumor resection, a high Karnofsky performance status and a young age at diagnosis are considered as clinical factors for prolonged survival (11), also the location and whether the subventricular zone is affected or not might have an influence for progression and survival (12). Furthermore, for several patients, eligibility for a second surgery after tumor recurrence, is an option associated with prolonged OS (13).

On the molecular level, some expression signatures, mutations, chromosomal aberrations and *MGMT* methylation status are deemed to be factors influencing therapy response and survival (14-16), but no common profile indicating LTS is found for this highly heterogeneous tumor entity (17). In contrary, some biomarkers like *MGMT* methylation are controversially discussed for outcome prediction ability (18).

Although several studies describe potential molecular features in connection with survival or prognosis (14,15), studies analyzing microRNA (miR) profiles are quite rare and mostly not concordant, describing different sets of miRs to be differentially expressed and therefore considered as potential biomarkers for survival (19-23).

MiRs are short non-coding RNAs of ~22 nt length which derive from long primary transcripts which are matured in a multistep process. They influence the transcription and translation of targeted genes and are often deregulated in any kind

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of cancer (24), also in glioblastoma (25). Depending on the tissue, kind of dysregulation and affected targets, they have oncogenic and/or tumor suppressive character (26). They are considered as putative targets for directed therapies consisting of miR-mimics for upregulating repressed miRs, or anti-miRs or miR-sponges to suppress overexpressed miRs (27-29).

The aim of this study is to compare miR expression profiles of short-time and long-time survivors to determine, whether there are survival-associated signatures. miRs showing significant differential expression between the survival groups are deemed as candidates for further analysis regarding potential target genes and influence on tumor behavior. These findings could contribute to more knowledge and deeper insights on molecular features of this highly complex and heterogeneous disease.

Identifying differentially expressed miRs and their potential target genes can give clues to factors affecting OS, which, in turn, could be interesting candidates for developing specific therapeutic approaches.

## Materials and methods

**Patient samples.** Formalin-fixed paraffin-embedded (FFPE) samples of GBM patients were retrieved from the archive of the Institute of Pathology of the University Medicine Rostock. Tissue was fixed in 4% neutral buffered formalin (Grimm) and incubated overnight at room temperature. Afterwards, it was embedded in molten paraffin (Merck).

Inclusion criteria were diagnosed GBM WHO Grade IV, sufficient availability of material and tumor content. For this study, the number of patient samples for array-based microRNA profile screening was limited, so from all potential available samples, initially 24 were arbitrarily selected to obtain a balanced distribution of sex and survival. One sample had to be removed afterwards due to issues of data quality and completeness, so a total of 23 patients were suitable for analysis.

Patient data (sex, age at diagnosis, OS, kind of surgery, therapeutic treatments) were obtained from the department of neurosurgery and patients were assigned to two groups: LTS and short-term survivor (STS) using the median OS of the cohort as discriminator. Molecular characteristics (*EGFR* amplification, mutation status of *IDH1* and *IDH2*) were obtained by earlier projects and/or routine diagnostic procedures.

Specimen collection was conducted in accordance with the ethics guidelines for the use of human material, approved by the Ethics Committee of the University of Rostock (Reference no. A 2009/34) and with informed written consent from all patients prior to surgery.

**MicroRNA extraction.** For microRNA extraction from 10  $\mu$ m FFPE sections, the miRNeasy FFPE-Kit (Qiagen) was used following the manufacturer's protocol. Concentration of extracted RNA was determined with a Nanodrop spectrometer (Peglab).

**miR screening arrays.** Analysis of microRNA expression was performed using the Nanostring nCounter System with the Human v3 miRNA assay (Nanostring). The analysis

procedure was performed with 250 ng per sample by a service provider lab (Transcriptome and Genome Analysis Laboratory (TAL), Microarray and Deep-Sequencing Facility, University Medicine Göttingen) and analyzed by the authors with the nSolver 4.0 software (Nanostring) using standard settings for background subtraction and housekeeping genes-based normalization. Group-wise comparison (LTS vs. STS) was performed, delivering fold change and p-values. Results were visualized as boxplots generated by BoxPlotR (<http://shiny.chemgrid.org/boxplotr/>).

**Determination of miR-targets.** For determination of potential target genes of miR-130b-3p, miR-146b-5p and miR-148a-3p, targeted literature research was performed looking up PubMed (<https://pubmed.ncbi.nlm.nih.gov>) for corresponding papers using search strings including the miR-name and 'glioblastoma'. Furthermore, the online microRNA-target interaction database miRTarBase (<http://mirtarbase.cuhk.edu.cn>) was consulted for predicted potential targets of these three miRs.

**Immunohistochemistry.** Protein expression analysis for PTEN and TRAF6 was performed by immunohistochemistry using 2  $\mu$ m thick FFPE sections on coated glass slides (Dako). For deparaffinization, rehydration and antigen demasking, slides were incubated for 20 min at 97°C and pH 9 in EnVision FLEX Target Retrieval Solution, high pH (pH 9) (Dako).

For slide processing, an automatic IHC system, AutostainerLink48 (Dako) was used, according to the following (routine) protocol. All steps were performed at room temperature. Slides were rinsed with EnVision FLEX Wash Buffer (Dako). To reduce unspecific background staining, slides were incubated for 5 min with 100  $\mu$ l of EnVision FLEX Peroxidase-Blocking Reagent, ready-to-use (Dako), a phosphate buffer containing H<sub>2</sub>O<sub>2</sub>, 15 mMol NaN<sub>3</sub> and detergent, and rinsed afterwards with wash buffer. For PTEN, a monoclonal (clone 6H2.1) mouse-anti human PTEN antibody (Dako, cat. no. M3627) was used, dilution 1:100 in EnVision FLEX Antibody Diluent (Dako). For TRAF6, a monoclonal (clone EP592Y) rabbit-anti human TRAF6 antibody (Abcam; cat. no. ab40675), dilution 1:50, was used. Slides were incubated with 100  $\mu$ l of first antibodies for 20 min. and rinsed afterwards with wash buffer. For TRAF6, a signal enhancement step was inserted incubating slides for 15 min with 100  $\mu$ l of EnVision FLEX+ Rabbit (LINKER), ready-to-use (Dako, cat. no. SM805). As secondary reagent, EnVision FLEX/HRP, ready-to-use (Dako, cat. no. SM802), was used, containing dextran coupled with peroxidase molecules and goat secondary antibodies against rabbit and mouse immunoglobulins. Slides were incubated with 100  $\mu$ l thereof for 20 min and rinsed afterwards with wash buffer. As substrate working solution 1 drop EnVision FLEX DAB+ Chromogen (Dako, cat. no. DM827) was mixed with 1 ml EnVision FLEX Substrate Buffer (Dako, cat. no. SM803). 200  $\mu$ l thereof were applied to each slide. Slides were incubated for 10 min and rinsed with wash buffer afterwards. Finally, for counter staining, 100  $\mu$ l of EnVision FLEX Hematoxylin (Dako, cat. no. SM806) were applied and slides were incubated for 5 min. Slides were rinsed with deionized water, washed for 5 min with wash buffer and rinsed once more with water. Slides were covered after dehydration

Table I. Characteristics of analyzed patients.

Patient no.	Age at diagnosis (y)	OS (d)	Sex	IDH1 status	EGFR status	Surgery	Rtx	TMZ	Survival status
1	67.36	528	M	Wt	Amp	Gross-total resection	Yes	Yes	LTS
2	47.36	143	F	Wt	Amp	Subtotal	Yes	Yes	STS
3	47.72	427	M	Wt	Amp	Gross-total resection	Yes	Yes	LTS
4	73.00	297	F	Wt	Amp	Gross-total resection	Yes	No	STS
5	59.96	727	M	Wt	Amp	Gross-total resection	Yes	Yes	LTS
6	69.63	380	F	Wt	Amp	Gross-total resection	Yes	Yes	STS
7	50.05	2041	M	Wt	Amp	Gross-total resection	Yes	Yes	LTS
8	64.47	148	M	Wt	Amp	Gross-total resection	Yes	Yes	STS
9	76.46	883	F	Wt	Amp	Gross-total resection	Yes	No	LTS
10	69.35	148	F	Wt	Amp	Gross-total resection	Yes	Yes	STS
11	53.22	236	M	Wt	Amp	Subtotal	N/A	N/A	STS
12	57.18	687	F	Wt	Norm	Gross-total resection	Yes	Yes	LTS
13	59.75	357	F	Wt	Norm	Gross-total resection	Yes	Yes	STS
14	57.46	586	F	Wt	Norm	Gross-total resection	Yes	Yes	LTS
15	49.17	253	M	Wt	Norm	Gross-total resection	Yes	Yes	STS
16	77.23	777	F	Wt	Norm	Gross-total resection	Yes	Yes	LTS
17	53.63	129	M	Wt	Norm	Gross-total resection	Yes	No	STS
18	80.82	489	F	Wt	Norm	Gross-total resection	Yes	Yes	LTS
19	49.62	120	F	Wt	Norm	Gross-total resection	Yes	No	STS
20	66.29	55	M	Wt	Norm	Subtotal	Yes	No	STS
21	71.09	30	M	R132H	Norm	Subtotal	Yes	No	STS
22	70.50	397	F	Wt	Amp	Gross-total resection	Yes	No	LTS
23	56.74	871	M	Wt	Norm	Gross-total resection	Yes	Yes	LTS

y, years; OS, overall survival; d, days; wt, wild type; R132H, IDH1 R132H mutation; norm, normal copy number; amp, amplification; STS, short-term survivor; LTS, long-term survivor; Rtx, radiotherapy.

with ethanol and xylol with coverslips (epredia) and CV mount (Leica) as mounting medium using the automated Coverslipper (Dako).

Stained slides were scanned using a Panoramic Desk DWII slide scanner (3DHitech) with Panoramic Scanner 2.2.0 software (3DHitech). Visualization and analysis were performed using CaseViewer 2.4 software (3DHitech). For presentation, representative regions the magnification was set to 10x, and the chosen sections were exported as JPEG.

**Statistical analyses.** Significance of differential miR expression was determined using the nSolver Software 4.0 (Nanostring) applying a paired, two-tailed Student's t-test. Statistical analyses concerning OS and clinical data were performed using SPSS Statistics version 28 (IBM) using the Kaplan-Meier method. Significance was assumed for  $P \leq 0.05$ .

## Results

**Patients' characteristics.** Samples of 23 GBM patients were finally included in this study (Table I). Median age at diagnosis was 59.96 years (47.36–80.82 years). Median OS was 380 days (30–2041 days). Patients with OS > median OS were classified as LTS, the others as STS. 11 patients were male,

12 were female. All but one were wild type for *IDH1* R132 and 12 carried an *EGFR* amplification. Sex and *EGFR* amplification status were evenly distributed over both survival groups. 19 of 23 patients underwent gross-total resection and 4 had a subtotal resection. 22 patients received radiotherapy, for one patient it is unknown. 15 patients were treated with TMZ, 7 received no TMZ, for one patient it is unknown. Of the patients without TMZ therapy, five were grouped into the STS group and 2 into LTS. Regarding the clinical data, only for the type of surgery, a significant influence on OS could be observed ( $P=0.001$ ). No significant differences in OS were seen for age at diagnosis ( $P=0.566$ ), sex ( $P=0.927$ ), *EGFR* status ( $P=0.437$ ) and TMZ treatment ( $P=0.158$ ) (Fig. S1, Table II).

**miR expression profiling.** The miR-expression profiling by the Nanostring nCounter software revealed on the whole just moderate changes in miR expression comparing profiles of LTS and STS (Table SI) for most of the miRs analyzed. But some miRs outperformed the moderate fold change values, rendering them as potential candidates as significant targets. Finally, three miRs were chosen for further analysis: miR-130b-3p, downregulated in LTS, miR-146b-5p and miR-148a-5p, both upregulated in LTS (Table III, Fig. 1). All three of them fulfilled the requirements for being further

Table II. Overview of clinical parameters and their influence on overall survival.

Clinical parameter	Variables, n (%)	Influence on OS (P-value)
Age at diagnosis, years		0.566
<60	11 (47.83%)	
>60	12 (52.17%)	
Median age, years	59.96	
Age range, years	47.36-80.82	
Sex		0.927
Female	12 (52.17%)	
Male	11 (47.83%)	
EGFR status		0.437
EGFR normal	11 (52.17%)	
EGFR amplified	12 (47.83%)	
IDH1/2 status		ND
Wild-type	22 (95.65%)	
Mutated	1 (4.35%)	
Tmz		0.158
Yes	15 (65.2%)	
No	7 (30.4 %)	
Unknown:	1 (4.35%)	
Radiotherapy		ND
Yes	22 (95.65%)	
Unknown	1 (4.35%)	
Type of surgery		0.001
Gross-total	19 (82.6%)	
Subtotal	4 (17.4%)	

OS, overall survival; ND, not determined; TMZ, temozolomid; IDH1/2 status, mutation status of IDH1 and IDH2 genes.

considered: a top fold change value with high significance, and robust absolute expression values (normalized counts) in both groups. MiRs showing high, significant fold change values, but only very low counts, weren't considered further.

Although there are overlaps of expression values between the two survival groups (Fig. 1), a kind of signature can be seen: low miR-130b-3p expression and high expression of miR-146b-5p and miR-148a-3p for LTS; and vice versa for STS. High and low expression states are separated by the median expression values. Correlating survival and expression status for each case shows that 11/23 cases (5 LTS and 6 STS) completely correspond to this signature and another 6 cases (4 LTS and 2 STS) in 2 of 3 miRs. Another 6 cases (2 LTS and 4 STS) show just one miR but no case shows none (Fig. 2).

**Determination of potential target genes.** Targeted PubMed search and specific investigation of miRTarBase revealed several genes proposed to be potential targets of miRs 130b-3p, 146b-5p and 148a-3p (Table IV). These two following were chosen to start further analyses with: PTEN as target of miR-130b-3p and important TSG in GBM, and TRAF6 as target of miR-146b-5p, an invasion promoting factor. For miR-148a-3p the situation is more complex, as it is described

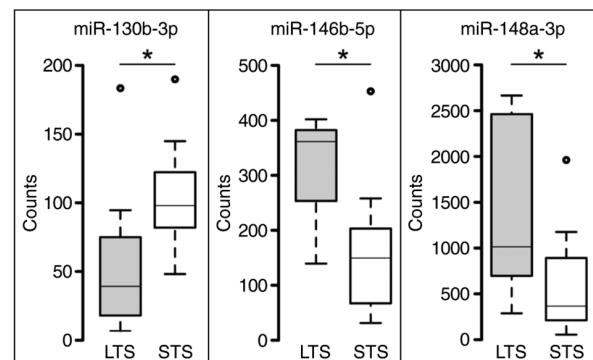


Figure 1. Boxplot representation of normalized expression values of miR-130b-3p, miR-146b-5p and miR-148a-3p, comparing LTS and STS groups. Center lines indicate medians, box limits indicate the 25 and 75th percentiles, whiskers extend 1.5x interquartile range from percentiles and dots represent outliers (generated using BoxPlotR; <http://shiny.chemgrid.org/boxplotr/>). \*P<0.05. miR, microRNA; STS, short-term survivor; LTS, long-term survivor.

to function either as oncogene or as TSG in GBM, so due to its ambivalent role, target studies were delayed.

**IHC analysis of PTEN and TRAF6.** Immunohistochemistry of PTEN and TRAF6 was successful in 22 of 23 cases.

Table III. Top 10 significantly differentially expressed miRs showing fold-change values of the LTS group ('true') compared with the STS group ('false').

Probe name	Accession no.	Fold-change LTS vs. STS	P-value
hsa-miR-146b-5p	MIMAT0002809	2.53	0.002
hsa-miR-130b-3p	MIMAT0000691	-2.72	0.007
hsa-miR-148a-3p	MIMAT0000243	3.20	0.007
hsa-miR-302b-3p	MIMAT0000715	1.78	0.011
hsa-miR-3065-5p	MIMAT0015066	3.90	0.012
hsa-miR-301b-3p	MIMAT0004958	-3.17	0.012
hsa-miR-887-3p	MIMAT0004951	2.24	0.013
hsa-miR-23a-3p	MIMAT0000078	1.94	0.017
hsa-miR-21-5p	MIMAT0000076	1.79	0.019
hsa-miR-142-3p	MIMAT0000434	2.02	0.019

Fold-change data of all miRs analyzed are presented in Table SI. STS, short-term survivor; LTS, long-term survivor; miR, microRNA.

Table IV. Literature and database predicted target genes for miR-130b-3p, miR-146b-5p and miR 148a-3p.

miR	Literature	miRTarBase
miR-130b-3p	PTEN (31); PPAR $\gamma$ (30); MST, SAV1 (32)	RUNX3, ZEB1, IGF1, TP53INP1
miR-146b-5p	TRAF6 (40,41); MMP16 (37); EGFR (34)	TRAF6, IRAK1, MMP16, KIT, EGFR
miR-148a-3p	ITGA9 (49); DLGAP1 (46); MIG, BIM (52)	DNMT3B, DNMT1, IKBKB, MMP7

miR, microRNA.



Figure 2. Visualization of the signature model miR-130b-3p low, miR-146b-5p high, miR-148a-3p high or vice versa. Each column represents a patient, and the number indicates for how many of the three miRNAs the model statement is true. Blue=long-term survivor; yellow=short-term survivor; green=low expression; red=high expression. miR, microRNA.

Although IHC is not considered as a quantitative method, it is observable, that a partial loss or decrease of PTEN expression occurs in some cases, mostly in samples with a higher miR-130b expression (Fig. 3), especially samples #11, #10 and #1.

Similarly, for TRAF6, a reduction or partial loss in expression is observable predominantly in samples with higher miR-146b expression (Fig. 4), especially samples #4, #1 and #5.

In summary, PTEN and TRAF6 could be considered as putative targets of miR-130b and miR-146b, respectively, at least in cases with higher miR expression levels.

## Discussion

This study shows a significant differential miR expression profile comparing LTS and STS, highlighting miR-130b-3p, miR-146b-5p and miR-148a-3p as most significantly differentially expressed miRNAs. As the definition of LTS is non-uniform throughout the literature, in our study, the median OS of the cohort (380 days) was used as delimiter to split the cohort in two more or less even groups. These data give the indication, that miR-130b-3p acts as oncomir, while miR-146b-5p and miR-148a-3p have tumor suppressor properties.



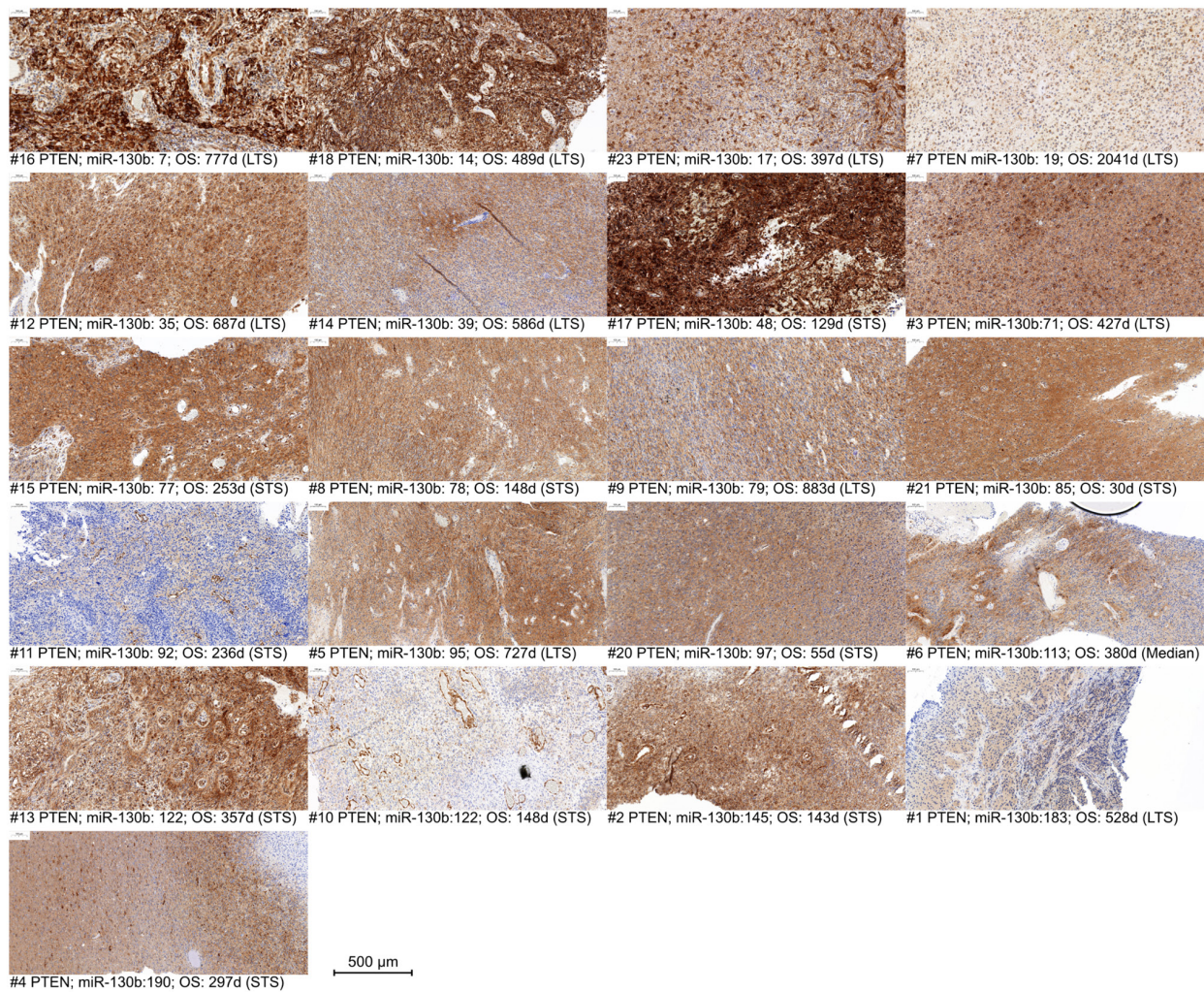


Figure 3. PTEN immunohistochemistry, sorted, from left to right, in an ascending order of miR-130b-3p counts days in patients #1 to 23. miR, microRNA; OS, overall survival; STS, short-term survivor; LTS, long-term survivor; d, days.

miR-130b is shown to be overexpressed in glioblastoma in contrast to non-neoplastic brain tissue, as well as in other tumor entities, and promotes cell proliferation by inhibiting *PPAR $\gamma$* , leading to decreased E-cadherin levels (30). Furthermore, it was shown, that suppression of miR-130b inhibits GBM cell proliferation and invasion and induces apoptosis via the PTEN/AKT signaling pathway. In this context, *PTEN* was shown to be a direct target of miR-130b (31). Another study shows more oncogenic properties of miR-130b, demonstrating that its upregulation enhances a stem cell-like phenotype by inactivating the Hippo signaling pathway with *MST1/2* and *SAVI* as direct targets (32). Earlier, miR-130b expression was associated with progression from lower to high grade glioma (33). Together with these, our data, showing a higher expression in STS samples, underpin the oncogenic character of miR-130b.

miR-146b-5p is linked with tumor suppressive properties. It is shown to target *EGFR* and hence, at least *in vitro*, reduces invasion and migration of glioma cells (34). In the cohort analyzed in our study, immunohistochemistry showed, that the *EGFR* expression was only dependent on the *EGFR* amplification status, but not on the survival group (data not shown). But for *in vitro* models it is shown that *EGFR* amplification is often lost during cultivation (35), except for special cell culture conditions (36).

Another target deemed to be regulated by miR-146b-5p is *MMP16*, whose inhibition leads to decreased migration and invasion (37,38). *MMP16* is a member of matrix metalloproteases, important factors for migration and metastasis as they contribute to proteolysis of the extracellular matrix, enabling tumor cell migration (39). *TRAF6* is another direct target of miR-146b-5p whose repression inhibits proliferation and progression, while promoting apoptosis. It is associated with a better prognosis (40). Furthermore, miR-146b-5p mediated *TRAF6* repression is considered to suppress TMZ resistance (41). *TRAF6* is correlated with a worse prognosis and oncogenic properties promoting invasion by upregulating another matrix-metalloprotease, *MMP9* (42). In context with our data these studies underpin the correlation of miR-146-5p, *TRAF6* expression and survival. Furthermore, miR-146b-5p inhibits GSCs and radioresistance by targeting *SMARCA5* (43) and the  $\beta$ -catenin pathway (44). Although most studies attest miR-146b-5p tumor suppressive properties, some oncogenic potential cannot be totally excluded, as its upregulation is also associated with GBM recurrence (45). But the data acquired in our own study are rather concordant with the described tumor suppressive properties.

For miR-148a, its role in GBM seems to be ambivalent, according to the current literature. Several studies nominate



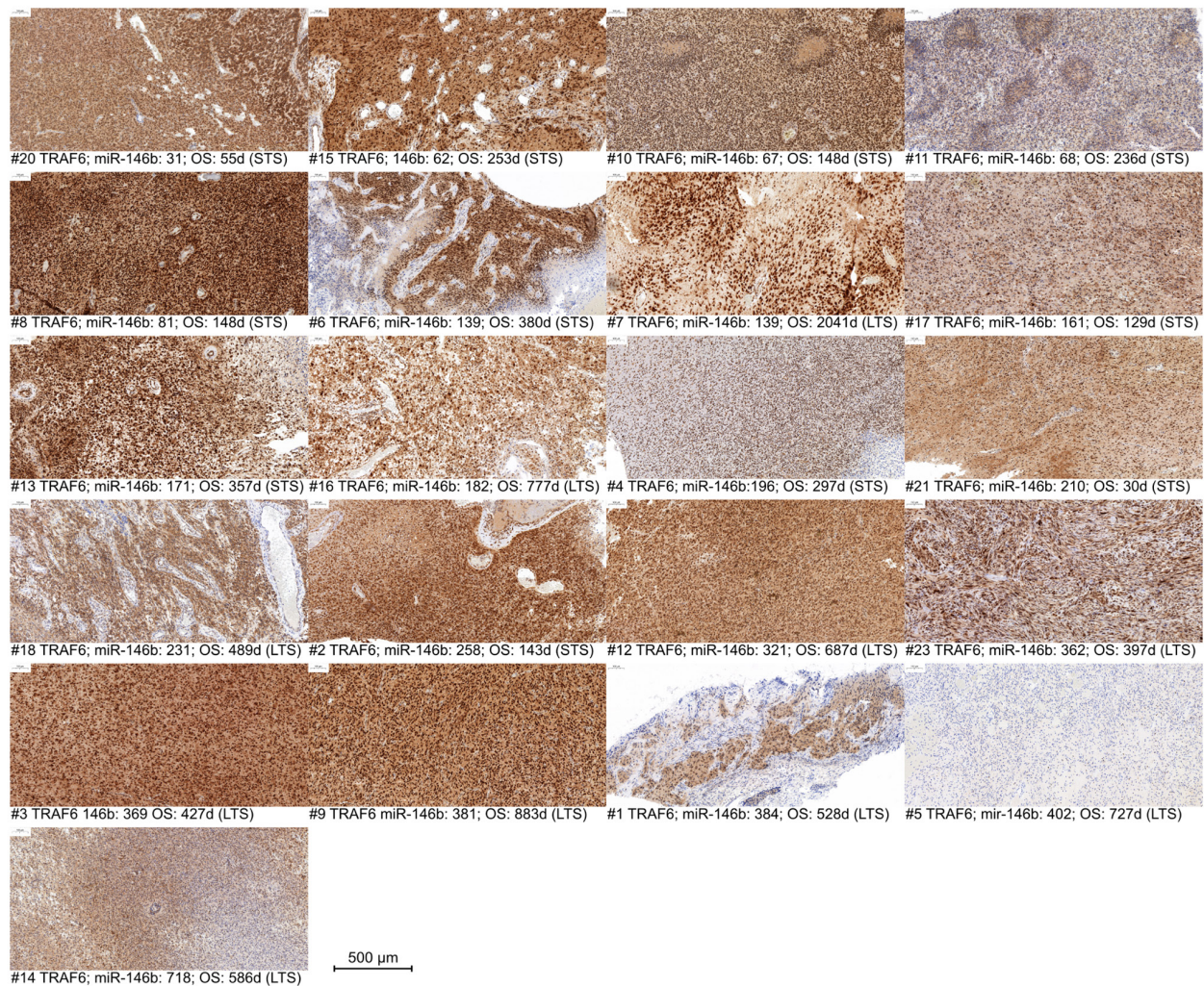


Figure 4. TRAF6 immunohistochemistry, sorted, from left to right, in an ascending order of miR-146b-5p counts in patients #1 to 23. miR, microRNA; OS, overall survival; STS, short-term survivor; LTS, long-term survivor; d, days; TRAF6, TNF receptor-associated factor 6.

different targets whose repression depict the oncogenic character of miR-148a, where others describe tumor suppressive properties. Repression of *DLGAP1* leads to loss of cell polarity, therefore promoting growth, migration, invasion and EMT (46). Overexpression of miR-148a deemed to be induced by NFκB and targeting *QKI* and *SKP1* activates TGFβ signaling and is associated with progression and augmented tumor aggressiveness (47). In a TCGA based study, it is shown that miR-148a targets *FIH1*, an *HIF1* inhibitor and therefore promotes *HIF1α* and NOTCH1 signaling, enhancing vascularization, growth and survival (48). But also the opposite effect on vascularization is described, as miR-148a has shown to have tumor suppressive properties targeting *ITGA9* (49), a cell adhesion factor involved in NOTCH1 controlled vascularization, leading to decreased angiogenesis (50). More oncogenic properties are demonstrated with targets like *GADD45A*, whose repression stimulates β-catenin and *MMP9*, promoting invasion, migration and stemness (51). Also positively influencing EGFR activity by targeting *MIG2* and inhibiting apoptosis by targeting *BIM* is described (52). Even exosomal delivery of miR-148a seems to have a positive effect to proliferation and progression by targeting *CADMI*, leading to STAT3 activation (53). On the other side, more tumor suppressive properties are known.

miR-148a targets *ROCK-1*, a factor promoting migration in several tumors (54). Even therapeutic effects are described, as *in vivo* experiments in mice show a prolonged survival when miR-148a is co-delivered with miR-296-5p by nanoparticles, targeting *OCT4* and *SOX2*, reducing stemness (29). Although studies describing oncogenic properties of miR-148a in GBM predominate, our own data show, that it is higher expressed in LTS, rendering it more to the tumor suppressive side.

Considering the exemplarily selected targets PTEN and TRAF6, the impression is not that clear. The expression of these targets was assessed by IHC. This method is not considered as quantitative and not sensitive enough to identify minor changes in expression which is characteristic for miR-mediated regulation. Also, there are much more factors influencing genes' translational activity. But the data show, that at least at the upper ranges of miR expression, a decrease in target gene expression is observable, but there's no linearity.

To elucidate the downstream effects of differential miR expression, identify the real target genes in these tumors, and observe the effects on tumor cell behavior, functional approaches with GBM *in vitro* models using transfection of miR mimics or antagomiRs, followed by analyses on different levels are needed.



This study is limited by a quite low number of patients analyzed and a small selection of potential target genes. To improve the value of robustness of the data, a consecutive study based on the present data should include a higher number of patients. Furthermore, next to functional studies, the selection of potential target genes should be extended to elucidate more details of the molecular mechanisms of tumor behavior underlying the outcome. Also, the study is carried out on FFPE material, which offers lesser quality and opportunities for deeper analysis. Fresh material would serve as a source better quality nucleic acids and proteins for deeper molecular analysis, and would even offer the opportunity to be taken in culture to provide cell lines or xenografts for *in vitro* or *in vivo* studies.

In summary, this study shows a significant correlation of differential miR-expression and survival status. It emphasizes the role of regulatory components/epigenetic factors, in this case microRNAs, within the complex interactions determining tumor behavior and outcome. These results hopefully help to clarify the so far widely unconcordant approaches to determine outcome predicting biomarkers. Although the regulatory impact to the few selected, potential miR target genes is rather low, the connection between miR expression and survival is significant. These data provide a good basis for further, functional studies.

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### Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

### Authors' contributions

BS designed the study, acquired, analyzed and interpreted data and wrote the manuscript. NL analyzed and interpreted immunohistochemistry data. AZ analyzed and interpreted immunohistochemistry data, contributed to the statistical analyses and critically edited the manuscript. CH acquired the patient data and substantially contributed to their analyses and interpretation, and critically edited the manuscript. AE provided patient material, substantially contributed to the concept of the study, and critically contributed to the manuscript. BS and AZ confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

### Ethics approval and consent to participate

The study was conducted in accordance with the Declaration of Helsinki, and approved by the Ethics Committee of the

University of Rostock (approval no. A2009/34). Informed consent was obtained from all subjects involved in the study.

### Patient consent for publication

Not applicable

### Competing interests

The authors declare that they have no competing interests.

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