RESEARCH PAPER

Expression of microRNAs miR-21 and miR-326 associated with HIF-1α regulation in neurospheres of glioblastoma submitted to ionizing radiation treatment

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ABSTRACT

Background: Glioblastoma is an incurable neoplasm. Its hypoxia mechanism associated with cancer stem cells (CSCs) demonstrates hypoxia-inducible factor 1α (HIF-1α) expression regulation, which is directly related to tumor malignancy. The aim of this study was to identify a possible tumor malignancy signature associated with regulation of HIF-1α by microRNAs miR-21 and miR-326 in the subpopulation of tumor stem cells which were irradiated by ion in primary culture of patients diagnosed with glioblastoma.

Materials and methods: We used cellular cultures from surgery biopsies of ten patients with glioblastoma. MicroRNA expressions were analyzed through real-time polymerase chain reaction (PCR) and correlated with mortality and recurrence. The ROC curve displayed the cutoff point of the respective microRNAs in relation to the clinical prognosis, separating them by group.

Results: The miR-21 addressed high level of expression in the irradiated neurosphere group (p = 0.0028). However, miR-21 was not associated with recurrence and mortality. miR-326 can be associated with tumoral recurrence (p = 0.032) in both groups; every 0.5 units of miR-326 increased the chances of recurrence by 1,024 (2.4%).

Conclusion: The high expression of miR-21 in the irradiated group suggests its role in the regulation of HIF-1α and in the radioresistant neurospheres. miR-326 increased the chances of recurrence in both groups, also demonstrating that positive regulation from miR-326 does not depend on ionizing radiation treatment.

Key words: glioblastoma; ionizing radiation; microRNAs

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Introduction

Glioblastoma is an incurable neoplasm. Chemoradiotherapy protocols have little effect on survival rates [1].

Therapies based on intrinsic characteristics of tumor stem cells (TSCs) have shown potential results, but the plasticity of the stem state inhypoxia detaches these cells of signals from their niches [2].

Intratumoral hypoxia promotes cancer's progression from hypoxia-inducible factors (HIFs). Signals from oncogenic transducing pathways regulate the plurality of CSCs and form oncospheres with high mitogenic rates [3].

Cellular bioenergetics is extensively remodeled by intratumoral hypoxia, causing changes in mitochondria oxidation and glycolytic metabolism. When added to an acidic microenvironment, numerous adaptations create favorable conditions for tumor aggression, such as modulations to microRNA expression profiles [3].

Resistance to radiation of glioblastoma is based on HIF-1α regulation. By establishing cooperation with other signaling pathways, the regulation of HIF-1α affects clonogenicity, DNA repair, and cell survival [4].

In severe hypoxic physiological conditions, normal neural progenitors express HIF-1α. This limits therapeutic efforts and reinforces the relevance of studies that analyze the expression of microRNAs, such as oncomiR miR-21 and anti-oncomiR miR-326. Both target HIF-1α regulation in the subpopulation of glioblastoma CSCs submitted to ionizing radiation [3].

miR-21 is substantially considered as oncomiR in glioblastoma. Its high expression is associated with a poor prognosis since, under hypoxic conditions, it can increase the proliferative rate in neural stem cells. However, it has been attested that its overexpression provides pro-differentiation into CSCs, suggesting that miR-21 inhibition could trigger tumor recurrences [3, 5].

The miR-326 and ARRB1 gene are highly expressed in differentiated cells, as they induce cell cycle arrest; other studies indicate that miR-326 induces interleukins 17 (IL-17) by blocking Ets-1, recruits TCD8+ cells promoting cytotoxic activities, in addition, miR-326 directly affects Hh signaling by establishing Smo as a target and reduces Notch pathways 1 and 2; therefore, miR-326 is associated with the capacity for self-renewal and differentiation of CD133+ glioma stem cells and compromises metabolic activity by reducing energy synthesis and cell survival, respectively. Therefore, suggesting that miR-326 may be a marker of glioma aggressiveness [6–9].

Therefore, this study aims to evaluate the expression of microRNAs: miR-21 and miR-326 in a primary culture of patients diagnosed with glioblastoma. We aim to identify a possible tumor malignancy signature associated with regulation of HIF-1α by microRNAs in the subpopulation of tumor stem cells with radiation ionizing.

Materials and methods

The present work was developed at the Molecular Biology Laboratory of the Department of Surgery and Anatomy of the Ribeirão Preto Medical School (FMRP-USP).

Primary samples

In this work, we used cell cultures from ten adult patients diagnosed with glioblastoma (glioma grade IV, WHO) at the Neurosurgery Division of the Clinical Hospital, Ribeirão Preto Medical School, University of São Paulo, from August 2014 to April 2015. This project was approved by the Research Ethics Committee of Hospital das Clínicas of Ribeirão Preto (protocol nº 17802/2015) (Tab. 1).

Processing and cell cultures

Fresh glioblastoma samples were washed and minced in PBS (1×). This was followed by enzymatic dissociation using collagenase-IV 100 U/mL (Gibco). For the establishment of the neurospheres culture, the cells were suspended in DMEM/F12, with growing factors of EGF and FGF [(20 ng/μL), Life Technologies Corporation, Gaithersburg, MD, USA], and 1% of penicillin. Whereas, for the establishment of the adhered cells culture, these were suspended in DMEM/F12, 10% of bovine fetal se-

Table 1. Demographic's characteristics: tumor site and surgical resection

	Tumor site	Surgical resection
GM1[R]	Temporal left	Subtotal
GM2	Temporal right	Total
GM3[R]	Temporal right	Total
GM4	Parietal left	Subtotal
GM5	Temporal and parietal right	Subtotal
GM6[R]	Superior-medial frontal gyrus left	Total
GM7	Frontal left	Total
GM8[R]	Parietal right	Total
GM9[R]	Parietal left	Subtotal
GM10	Fronto-parietal in dimidio	Subtotal

[R]relapsed patients

rum (Life Technologies Corporation, Gaithersburg, MD, USA) and 1% of penicillin.

The cells were put in culture bottles of 75 cm^2 (TPP®) and inside an incubator with 5% of CO_2 andin 37°C. The culture environment was changed every 48 hours, and the cells were kept in these conditions for about two weeks, until they reached the confluence needed for the execution of the experiments.

Experimental groups

After the primary cultures of the ten patients were divided in two initial groups (neurospheres and adhered cells), the infringed experimental procedures were described solely for the neurosphere group:

- Control group (C): 10^5 cells were sown and cultivated for 48 hours, and then collected for analysis without treatment [10].
- Group treated with ionizing radiation (R): 10^5 were seeded 24 hours before treatment with ionizing radiation, with a dose rate of 2.0 Gy/min, (60Co source, Unit Gammatron S-80, Siemens, 1.25 MeV, HC-FMRP/USP), for a total dose of 14 Gy. Collection occurred 48 hours after treatment [10].

Cell viability assay

The cell viability assay was assessed by a dye exclusion method with trypan blue. The cells were briefly re-suspended in 1000 μL of **tumor brain stem cell medium**, and trypan blue solution (0.4%) was then added to the cells in equal volume. After 15-20 minutes of incubation, we counted the cells using a hemacytometer at room temperature. After counting, the ratio of viable cells to the total number of cells was calculated and recorded.

RNA isolation and real-time polymerase chain reaction

Total RNA was extracted using Trizol reagent (Applied Biosystems, Foster City, United States) in accordance with the manufacturer's instructions. Considering the preparation of real-time polymerase chain reaction (PCR), reverse transcription of RNA samples was performed using the High-Capacity cDNA kit (Applied Biosystems).

The selected microRNAs were analyzed by the tool *MirPath V.3*, available on the digital platform Diana Tools (http://snf-515788.vm.okeanos. grnet.

gr/). This tool identifies the signals' pathways that come from the database KEGG (http://www.genome.jp/kegg/pathway.html) which are potentially regulated by the expression of the microRNAs. Thus, in the analysis, it was possible to identify the microRNAs: miR-21-5p and miR-326 which presented as targets the gene HIF-1α. The targets were confirmed in the database of miRTarBase (http://mirtarbase.mbc.nctu.edu.tw/php/download.php).

The cDNA was amplified with quantitative real time polymerase chain reaction (q-PCR) using TaqMan Master Mix (Applied Biosystems) for the reaction of microRNAs. The U6 gene was used as an endogenous control (housekeeping) for the reaction of the microRNA. The PCR conditions included pre-heating at 50°C for two minutes, denaturation at 95°C for ten minutes, and 50 cycles of amplification and quantification (15 seconds at 95°C, and one minute at 60°C). All reactions were performed doubly and analyzed with the 7500 Sequence Detection System apparatus (Applied Biosystems). The data were analyzed using ABI-7500 SDS software. Dissociation curves were performed (melting curves) after amplification by RQ-PCR. The samples that showed dissociation curves with different temperatures or more than one point of dissociation in the same sample were not considered and repeated.

Statistical analysis

Regression coefficients and log transformation application, Poisson regression model, and ROC curve, were used to analyze microRNAs expressions by PCR in real time, relative risks, and predictive value of the studied microRNAs, respectively. All the presented graphs were made with the assistance from software R, version 4.0.0. Analyses were performed with the assistance from SAS 9.2 software. P values smaller than 0.05 were considered to be statically significant.

Results

When analyzing the cell viability of tumor samples with the proposed protocol and isolated radiation by ion, no statistically significant differences were identified between neurospheres from the experimental groups.

miR-326 was overexpressed in the group of neurospheres submitted to ionizing radiation
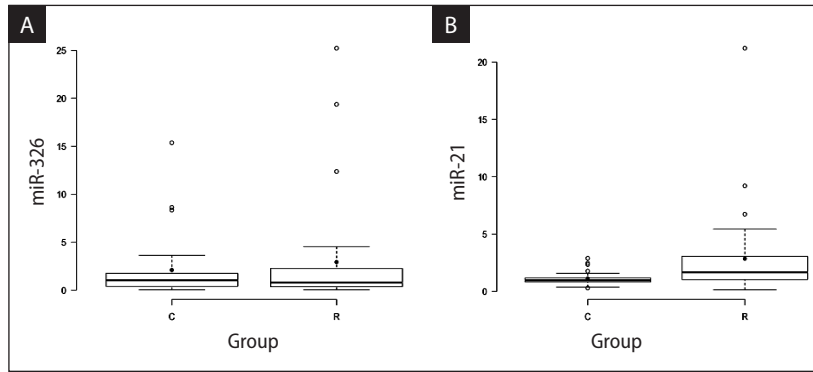
Figure 1. A. Representation of miR-326 expression in neurospheres treated with ionizing radiation. It can be observed that there was no statistical difference (p = 0.6309); **B.** Representation of miR-21 expression in neurospheres treated with ionizing radiation. It can be noticed that there was a significant increase in the irradiated neurospheres (R) in comparison to the control group (C) (p = 0.0028)
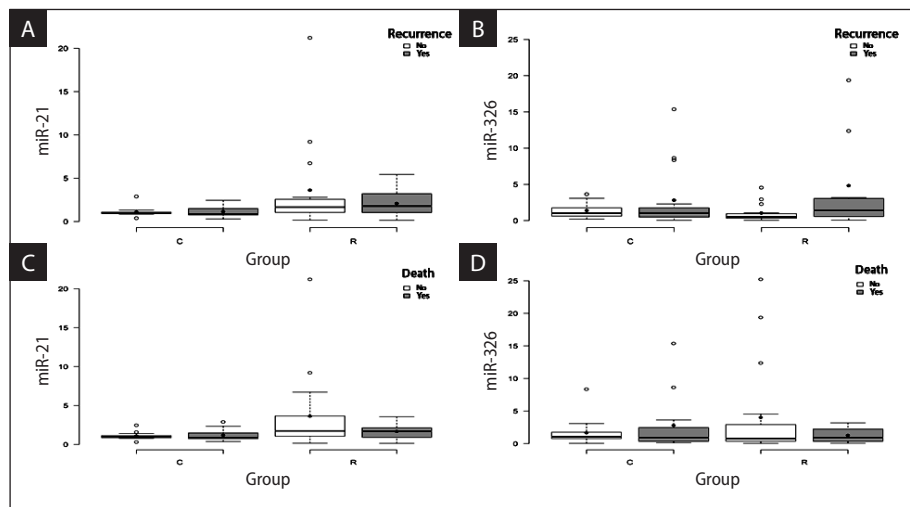

Figure 2. AB. Representation of the median regarding the recurrence for microRNAs miR-21 and miR-326 expressed in neurospheres relative to the groups studied; **CD.** Representation of the median regarding mortality of microRNAs miR-21 and miR-326 expressed relative to neurospheres in the groups studied

treatment when compared to the control group. However, there was no statistically significant difference (p = 0.6309) (Fig. 1A). miR-21 did show statistical difference in the group of neurospheres submitted to ionizing radiation treatment (p = 0.0028) when compared to the control group (Fig. 1B).

For every 0.5 units of miR-21, the risk of recurrence increased by 1,004. Thus, there is practically no association between the variables, as the recurrence is not dependent on the occurrence of miR-21 in both groups (Tab. 2A, Fig. 3A, 4A). For every 0.5 units of miR-326, the risk of recurrence increased by 1,024 (2.4%), demonstrating an association between the variables miR-326 and recurrence

(p = 0.032) in both groups (Tab. 2A, Fig. 3B, 4B). No associations were verified between the variables miR-21 and miR-326 in relation to mortality in both groups (Tab. 2B, Fig. 3CD, 4CD).

The confidence interval shows, with 95% confidence level, the real area under the curve (AUC). Thus, the elevated confidence intervals do not consider any predictions of the studied microRNAs in relation to recurrence and mortality in both experimental groups, despite a near ideal AUC result (Figure 4, 5). The reliability of the estimation also becomes invalid when the AUC is close to the random line, as this indicates a mischaracterization of these microRNAs as a predictive responsive to recurrence and mortality (Fig. 4CD). Consequent-

Table 2. Estimation of the relative recurrence risk and the relative risk of mortality

Effect	RR	95% CI		p-value
Relative recurrence risk				
miR-21	1.004	0.917	1.099	0.934
miR-21 (Group C)	1.050	0.908	1.215	0.509
miR-21 (Group R)	0.960	0.878	1.049	0.362
miR-326	1.024	1.002	1.047	0.032
miR-326 (Group C)	1.027	1.001	1.055	0.046
miR-326 (Group R)	1.021	1.003	1.039	0.022
Relative risk of mortality				
miR-21	0.990	0.880	1.114	0.872
miR-21 (Group C)	1.088	0.933	1.268	0.284
miR-21 (Group R)	0.902	0.794	1.025	0.114
miR-326	0.986	0.948	1.025	0.469
miR-326 (Group C)	1.027	0.997	1.058	0.076
miR-326 (Group R)	0.946	0.887	1.009	0.093

RR — relative risk; CI — confidence interval
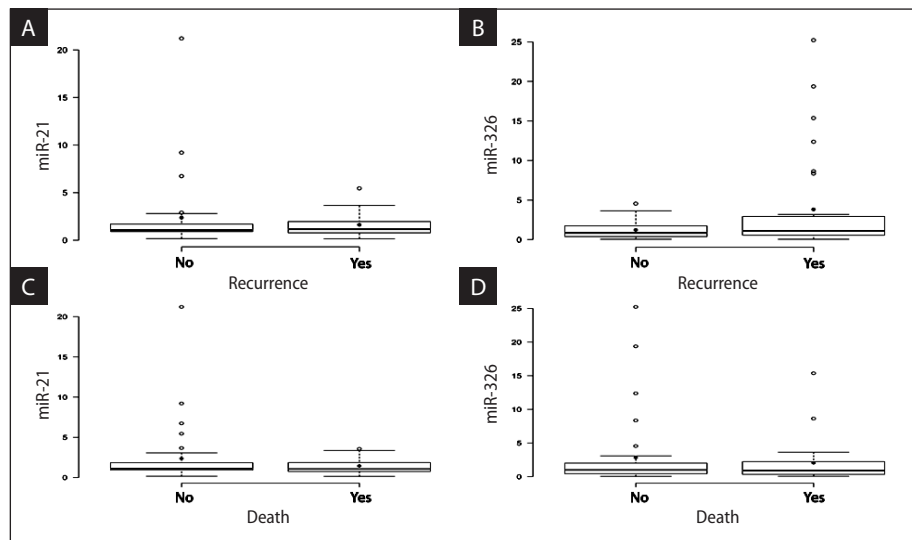
Figure 3. AB. Representation of the median regarding the recurrence of microRNAs miR-21 and miR-326 expressed relative to the neurospheres; **CD.** Representation of the median regarding mortality of microRNAs miR-21 and miR-326

ly, it was not possible to establish cutoff points for sensitivity or specificity (Tab. 5, 6).

Discussion

miR-21 was overexpressed in the group of neurospheres submitted to ionizing radiation treatment (p = 0.0028) when compared to the control group. It was possible to affirm that miR-21 in normoxic cancer stem cells is one of the interfering factors from radio resistance, which is in agreement with previous research.

Hypoxia and the Warburg Effect create an acidic microenvironment that promotes the release of exosomes. In the present study, the neurospheres grew in normoxic conditions, and elevated miR-21 levels in the irradiated group were recorded. These findings suggest radiotherapy eliminated tumor
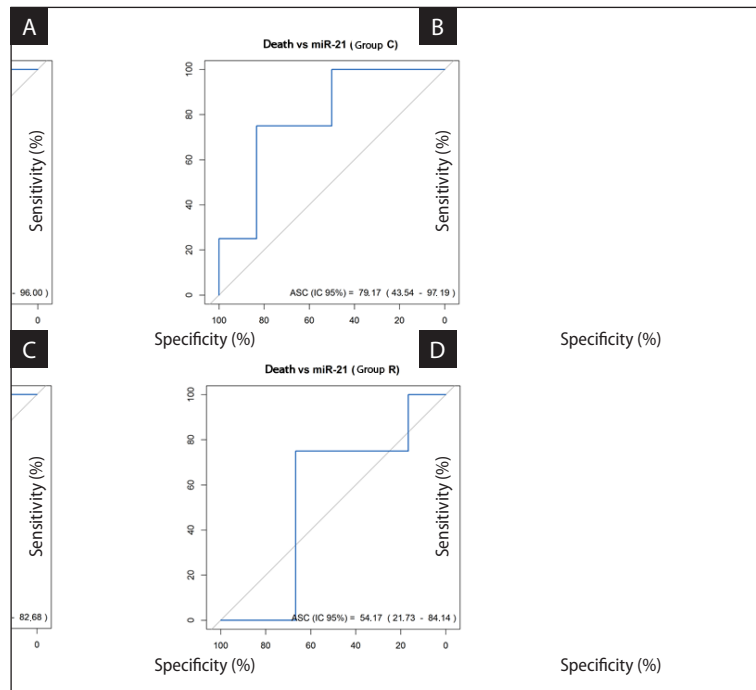
Figure 4. AB. ROC curves of miR-21 as a predictive value for recurrence and mortality relative to the neurosphere control group; **CD.** ROC curves of miR-21 as a predictive value for recurrence and mortality related to the irradiated neurosphere group
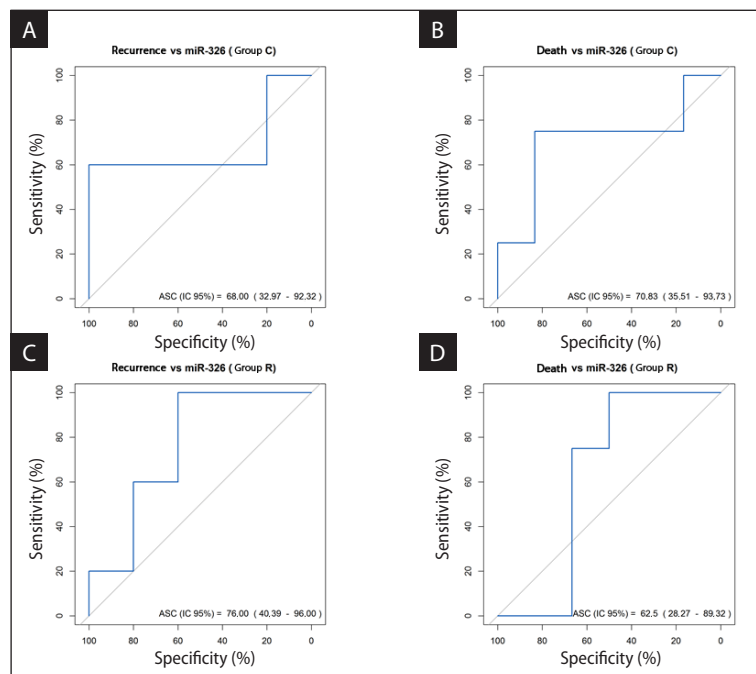

Figure 5. AB. ROC curves of miR-326 as a predictive value of recurrence and mortality relative to the neurosphere control group; **CD.** ROC curves of miR-326 as a predictive value of recurrence relative to the irradiated neurosphere group

cells and reduced miR-21 levels as a consequence. Thus, ionizing radiation positively corroborated with tumor progression.

Other studies that used hypoxic growth conditions have reported that the acid microenvironment triggers the activation of HIF-1α and HIF-2α, stim-

Table 3. Description of the microRNAs miR-21 and miR-326 regarding experimental groups, mortality, and recurrence obtained from clinical records after surgical resection and chemoradiotherapy protocol

Group	Death	Variables	n	Mean	SD	Minimum	Median	Maximum
C	No	Fold miR-21	18	1.08	0.44	0.29	0.98	2.46
		Fold miR-326	18	1.64	1.88	0.05	1.04	8.34
	Yes	Fold miR-21	12	1.18	0.76	0.38	0.89	2.89
		Fold miR-326	12	2.79	4.62	0.12	0.94	15.36
R	No	Fold miR-21	18	3.63	4.98	0.17	1.75	21.21
		Fold miR-326	18	4.01	7.31	0.04	0.8	25.22
	Yes	Fold miR-21	12	1.67	1.04	0.14	1.68	3.56
		Fold miR-326	12	1.27	1.12	0.07	0.93	3.17
Group	Recurrence	Variables	n	Mean	SD	Minimum	Median	Maximum
C	No	Fold miR-21	15	1.08	0.54	0.38	0.97	2.89
		Fold miR-326	15	1.39	1.07	0.20	1.02	3.62
	Yes	Fold miR-21	15	1.15	0.64	0.29	0.87	2.46
		Fold miR-326	15	2.81	4.42	0.05	1.05	15.36
R	No	Fold miR-21	15	3.61	5.44	0.17	1.67	21.21
		Fold miR-326	15	1.03	1.26	0.04	0.5	4.53
	Yes	Fold miR-21	15	2.07	1.45	0.14	1.78	5.43
		Fold miR-326	15	4.81	7.78	0.07	1.43	25.22

SD — standard deviation

Table 4. A. Descriptive statistics regarding solely the relapse variable of microRNAs miR-21 and miR-326; **B.** Descriptive statistics of microRNAs miR-21 and miR-326 regarding solely the mortality variable

Death	Variables	n	Mean	SD	Minimum	Median	Maximum
No	Fold miR-21	36	2.35	3.72	0.17	1.12	21.21
	Fold miR-326	36	2.83	5.40	0.04	0.99	25.22
Yes	Fold miR-21	24	1.42	0.92	0.14	1.04	3.56
	Fold miR-326	24	2.03	3.38	0.07	0.94	15.36
Recurrence	Variables	n	Mean	SD	Minimum	Median	Maximum
No	Fold miR-21	30	2.35	4.01	0.17	1.05	21.21
	Fold miR-326	30	1.21	1.16	0.04	0.84	4.53
Yes	Fold miR-21	30	1.61	1.19	0.14	1.15	5.43
	Fold miR-326	30	3.81	6.30	0.05	1.11	25.22

SD — standard deviation

ulating the expression of exosomal miR-21. This substantially promotes the proliferation, migration, and invasion of hepatocellular carcinoma [11].

However, miR-21 was not associated with recurrence and mortality in the present study, as recurrence and mortality occurred independent of miR-21 in both groups.

It is suggested that miR-21 does not correlate with putative CSC markers, such as OCT4, SOX2, and CD133. However, the difficulty regarding molecular characterizations of OCT4, SOX2, and

CD133 do not exclude the possibility that miR-21 intervenes with the properties of the stem state, suggesting the pro-angiogenic role of miR-21 in glioblastoma. In addition, by immunohistochemistry, negative regulation of PTEN was not recorded in areas that were positive for miR-21, suggesting that miR-21 may not be the principal mechanism that elevated levels of HIF-1α and VEGF [12].

It is extremely rare for glioblastomas to metastasize systematically. However, extracellular vesicles from CSCs are directed to differentiated cells,

Table 5. AB. Cutoff point, sensitivity, and specificity of miR-21 expression in the neurosphere control group relative to recurrence and mortality, with area under the curve (AUC) = 76%, 95% confidence level (CI) = 40.39 – 96% and AUC = 79.17%, 95% CI = 43.54–97.19%, respectively. **CD.** Cutoff point, sensitivity, and specificity of miR-21 expression in the irradiated neurosphere group regarding the recurrence and mortality, with AUC = 52%, 95% CI = 20.14–82.68% and AUC = 54.17%, 95% CI = 21.73–84.14%, respectively

A	Criterion	Specificity (95% CI)	Sensitivity (95% CI)
	≥ 1.0003	0 (0.0–52.2)	100 (47.8–100.0)
	> 1.0003	20 (0.5–71.6)	100 (47.8–100.0)
	> 1.0045	40 (5.3–85.3)	100 (47.8–100.0)
	> 1.0068	**60 (14.7–94.7)**	**100 (47.8–100.0)**
	> 1.0078	60 (14.7–94.7)	80 (28.4–99.5)
	> 1.0195	80 (28.4–99.5)	80 (28.4–99.5)
	> 1.0582	80 (28.4–99.5)	60 (14.7–94.7)
	> 1.0905	80 (28.4–99.5)	40 (5.3–85.3)
	> 1.2158	80 (28.4–99.5)	20 (0.5–71.6)
	> 1.3829	80 (28.4–99.5)	0 (0.0–52.2)
	> 1.3918	100 (47.8–100.0)	0 (0.0–52.2)

B	Criterion	Specificity (95% CI)	Sensitivity (95% CI)
	≥ 0.7801	0 (0.0–52.2)	100 (47.8–100.0)
	> 0.7801	20 (0.5–71.6)	100 (47.8–100.0)
	> 1.0906	20 (0.5–71.6)	80 (28.4–99.5)
	> 1.2155	**40 (5.3–85.3)**	**80 (28.4–99.5)**
	> 1.5257	40 (5.3–85.3)	60 (14.7–94.7)
	> 1.8088	60 (14.7–94.7)	60 (14.7–94.7)
	> 1.8109	60 (14.7–94.7)	40 (5.3–85.3)
	> 1.8847	60 (14.7–94.7)	20 (0.5–71.6)
	> 1.8934	80 (28.4–99.5)	20 (0.5–71.6)
	> 4.0513	80 (28.4–99.5)	0 (0.0–52.2)
	> 12.3759	100 (47.8–100.0)	0 (0.0–52.2)

C	Criterion	Specificity (95% CI)	Sensitivity (95% CI)
	≥ 1.0003	0 (0.0–45.9)	100 (39.8–100.0)
	> 1.0003	16.67 (0.4–64.1)	100 (39.8–100.0)
	> 1.0045	33.33 (4.3–77.7)	100 (39.8–100.0)
	> 1.0068	50 (11.8–88.2)	100 (39.8–100.0)
	> 1.0078	50 (11.8–88.2)	75 (19.4–99.4)
	> 1.0195	66.67 (22.3–95.7)	75 (19.4–99.4)
	> 1.0582	**83.33 (35.9–99.6)**	**75 (19.4–99.4)**
	> 1.0905	83.33 (35.9–99.6)	50 (6.8–93.2)
	> 1.2158	83.33 (35.9–99.6)	25 (0.6–80.6)
	> 1.3829	100 (54.1–100.0)	25 (0.6–80.6)
	> 1.3918	100 (54.1–100.0)	0 (0.0–60.2)

D	Criterion	Specificity (95% CI)	Sensitivity (95% CI)
	≥ 0.7801	0 (0.0–45.9)	100 (39.8–100.0)
	> 0.7801	16.67 (0.4–64.1)	100 (39.8–100.0)
	> 1.0906	16.67 (0.4–64.1)	75 (19.4–99.4)
	> 1.2155	33.33 (4.3–77.7)	75 (19.4–99.4)
	> 1.5257	50 (11.8–88.2)	75 (19.4–99.4)
	> 1.8088	**66.67 (22.3–95.7)**	**75 (19.4–99.4)**
	> 1.8109	66.67 (22.3–95.7)	50 (6.8–93.2)
	> 1.8847	66.67 (22.3–95.7)	25 (0.6–80.6)
	> 1.8934	66.67 (22.3–95.7)	0 (0.0–60.2)
	> 4.0513	83.33 (35.9–99.6)	0 (0.0–60.2)
	> 12.3759	100 (54.1–100.0)	0 (0.0–60.2)

affecting their phenotype. But the contrary is also true. In glioblastoma, significant concentrations of miR-21 have been detected in the vesicular content, where microglia and monocytes are suppressed. microRNAs derived from extracellular vesicles are not randomly packaged, as they concentrate on only 31-64 target genes. In this scenario, PTEN is silenced too much, and PI3-AKT is activated. Hypoxia is also known to change the vesicular contents in transfer [13].

Studies have shown that miR-21 transferred though extracellular vesicles can deregulate specific mRNAs from wild-type microglia, *in vitro* and *in vivo*, as microglial proliferation increases after delivery of miR-21 by negatively regulating BTG2. This decreases the activity of cyclin D1 and reduces cellular proliferation by inhibiting the transition of the G1 phase to the S phase, as demonstrated in fibroblasts.

miR-326 expressed positive regulation, but it did not show significant statistical difference when compared to the ionizing radiation treatment group and the control group. However, evidence of an association between miR-326 and recurrence (p = 0.032) was observed in both groups; for every 0.5 units of miR-326, the risk of recurrence increased by 1,024 (2.4%). With this data, it is possible to stress that the expression of miR-326 does not depend on radiotherapy, as ionizing radiation treatment is expected to increase the levels of miR-326.

In biological processes, miR-326 is related to the ephrin receptor signaling pathway, axon orientation, and axonogenesis. However, it can also inhibit cell proliferation, migration, and invasion in various types of cancers through the regulation of various factors, such as KRAS, TWIST1, LIM, SH3,

Table 6. AB. Cutoff point, sensibility, and specificity of miR-326 expressed in control group neurospheres regarding the recurrence and mortality, with area under the curve (AUC) = 68%, 95% confidence level (CI) = 32.97–92.32% and AUC = 70.83%, 95% CI = 35.51–93.73%, respectively; **CD.** Cutoff point, sensibility, and specificity of miR-326 expressed in irradiated group neurospheres regarding the recurrence and mortality, with AUC = 68%, 95% CI = 40.39–96% and AUC = 62.5%, 95% CI = 28.27–89.32%, respectively

A	Criterion	Specificity (95% CI)	Sensitivity (95% CI)
	≥ 1.0002	0 (0.0–52.2)	100 (47.8–100.0)
	> 1.0002	20 (0.5–71.6)	100 (47.8–100.0)
	> 1.0095	20 (0.5–71.6)	80 (28.4–99.5)
	> 1.0176	20 (0.5–71.6)	60 (14.7–94.7)
	> 1.2031	40 (5.3–85.3)	60 (14.7–94.7)
	> 1.4283	60 (14.7–94.7)	60 (14.7–94.7)
	> 1.6145	80 (28.4–99.5)	60 (14.7–94.7)
	> 1.7190	**100 (47.8–100.0)**	**60 (14.7–94.7)**
	> 3.1000	100 (47.8–100.0)	40 (5.3–85.3)
	> 3.5588	100 (47.8–100.0)	20 (0.5–71.6)
	> 5.3439	100 (47.8–100.0)	0 (0.0–52.2)

B	Criterion	Specificity (95% CI)	Sensitivity (95% CI)
	≥ 0.2102	0 (0.0–52.2)	100 (47.8–100.0)
	> 0.2102	20 (0.5–71.6)	100 (47.8–100.0)
	> 0.4071	40 (5.3–85.3)	100 (47.8–100.0)
	> 0.7589	**60 (14.7–94.7)**	**100 (47.8–100.0)**
	> 0.9330	60 (14.7–94.7)	80 (28.4–99.5)
	> 1.0297	60 (14.7–94.7)	60 (14.7–94.7)
	> 1.0533	80 (28.4–99.5)	60 (14.7–94.7)
	> 1.1191	80 (28.4–99.5)	40 (5.3–85.3)
	> 1.9715	80 (28.4–99.5)	20 (0.5–71.6)
	> 2.7080	100 (47.8–100.0)	20 (0.5–71.6)
	> 18.9739	100 (47.8–100.0)	0 (0.0–52.2)

C	Criterion	Specificity (95% CI)	Sensitivity (95% CI)
	≥ 1.0002	0 (0.0–45.9)	100 (39.8–100.0)
	> 1.0002	16.67 (0.4–64.1)	100 (39.8–100.0)
	> 1.0095	16.67 (0.4–64.1)	75 (19.4–99.4)
	> 1.0176	33.33 (4.3–77.7)	75 (19.4–99.4)
	> 1.2031	50 (11.8–88.2)	75 (19.4–99.4)
	> 1.4283	66.67 (22.3–95.7)	75 (19.4–99.4)
	> 1.6145	**83.33 (35.9–99.6)**	**75 (19.4–99.4)**
	> 1.7190	83.33 (35.9–99.6)	50 (6.8–93.2)
	> 3.1000	83.33 (35.9–99.6)	25 (0.6–80.6)
	> 3.5588	100 (54.1–100.0)	25 (0.6–80.6)
	> 5.3439	100 (54.1–100.0)	0 (0.0–60.2)

D	Criterion	Specificity (95% CI)	Sensitivity (95% CI)
	≥ 0.2102	0 (0.0–45.9)	100 (39.8–100.0)
	> 0.2102	16.67 (0.4–64.1)	100 (39.8–100.0)
	> 0.4071	33.33 (4.3–77.7)	100 (39.8–100.0)
	> 0.7589	**50 (11.8–88.2)**	**100 (39.8–100.0)**
	> 0.9330	50 (11.8–88.2)	75 (19.4–99.4)
	> 1.0297	66.67 (22.3–95.7)	75 (19.4–99.4)
	> 1.0533	66.67 (22.3–95.7)	50 (6.8–93.2)
	> 1.1191	66.67 (22.3–95.7)	25 (0.6–80.6)
	> 1.9715	66.67 (22.3–95.7)	0 (0.0–60.2)
	> 2.7080	83.33 (35.9–99.6)	0 (0.0–60.2)
	> 18.9739	100 (54.1–100.0)	0 (0.0–60.2)

ELK1, among other combinatorial systems. Studies have discussed the possibility that the functions and concentrations of miR-326 vary in tumor stages, suggesting that increased levels of this microRNA may correlate with lower survival rates [15].

It is speculated that other molecular agents interact with miR-326 to positively regulate glioblastoma and establish miR-326 as a feedback function [16].

Contrary to the data displayed in this study, the low expression of miR-326 is frequently associated with metastatic development and lower survival rates, as it targets genes related to the activity of disintegrins, metalloproteases, and proteins involving nucleosome dynamics. However, these specific mechanisms have not yet been elucidated [17].

In studies with non-small cell lung cancer, the prognostic function of miR-326 has been correlated with HIF-1α overexpression [15].

Fibroblast growth factor 1 (FGF1) and nerve growth factor (NGF) regulate miR-326 expression. Consequently, this can repair blood vessels by promoting proliferation and invasion of endothelial cells and pericytes. In addition, tumor growth and chemoradiotherapy resistance are stimulated, as MAPK, PI3K, RAS, and JNK mitogenic pathways are signaled. The miR-326high expression, which occurs from decreases of FGF1 and NGF, inhibits malignant behavior. However, abnormal PI3 signaling results in the negative regulation of the miR-326 in glioblastomas [17, 18]. But the association between miR-326 and mortality rates was not verified in the current study.

Via the AKT-mTOR pathway, the PINK1 gene is involved in mitochondrial regulation, ATP generation, molecular oxygen consumption, ROS production, and anti-apoptotic and cytoprotective func-

tions. In addition, the low expression of the PINK1 gene decreases the synthesis of glial fibrillary acid protein (GFAP), an astrocyte marker. It is also related to the negative regulation of miR-326. Therefore, the low expression of miR-326 could be related to undifferentiated neural stem cells, as undifferentiated astrocytes demonstrate that PINK1 low expression is not demarcated by GFAP [19].

Proteins of the high mobility groups A1 (HMGA1) and A2 (HMGA2), implicated in tumor invasion and recurrence, are suggested as possible targets for miR-326. Bioinformatic approaches have detected that the oncogenic factor HOTAIR is also a target for miR-326, as its silencing effect significantly reduces fibroblastic growth factor 1 (FGF1) [18]. However, our data demonstrated that the overexpression of miR-326 is a function of the recurrence rate. Such results should encourage investment in new studies with high sample variability to demonstrate the most diverse signals of miR-326, possible target genes, and possible sovereignty over microRNAs that constitute epigenetics and the diverse phenomena of tumorigenesis.

Long noncoding RNAs (lncRNAs) can act as sponges to microRNAs, limiting their availability and reducing their regulatory effects. H19 high expression was demonstrated in glioblastomas, and angiogenesis and an increase of invasiveness were confirmed. This contradicts the overexpression of miR-326 in gliomas, and it results in decreased cell proliferation and an apoptotic increase [16].

The area under the curve (AUC) in C groups, in both microRNAs, is considerable. However, the small sample size does not allow us to stress adequate cutoff points for recurrence and death. However, tumor heterogeneity increases population variability. Therefore, a large CI would be an inherent aspect of malignancy in glioblastoma.

Solely from this study, it is not possible to define microRNAs miR-21 and miR-326 as prognostic markers for stage assessment in radiotherapy. The sample number must be increased in future studies so that cutoff points can reach maximum diagnostic and prognostic accuracy. The experimental design should also be applied to liquid biopsies from plasma and cerebrospinal fluid.

In the present study, cell growth occurred in normoxic conditions. However, studies have suggested that significant tumor growth occurs after radiotherapy in regions with high incidences of inter-

mittent vascular stasis. Therefore, cyclically hypoxic cells may be more radioresistant for modulating angiogenic processes, which also characterizes radioresistance to the tumor endothelium[20]. Future experiments should be performed in hypoxic conditions to allow for findings compatible with the pO_2 of solid tumors *in vivo*.

Our work confirms the role of miR-21 in the regulation of the subpopulation of tumor stem cells. Our results also reinforce that the concentration and function of microRNAs, such as miR-326, have a unique signature in different tumor stages, relapses, and responses to treatments. Most importantly, future research should investigate the diverse interferences that overlap the regulatory effects of tumor-suppressing microRNAs. Investment is needed in research that constitutes a therapeutic set based on maneuvers related to tumor oxygenation, degradation of HIF-1α e HIF-2α, inhibition of residual HIF-1α, and associating these factors with immunotherapy to silence glioblastoma malignancy.

Conclusion

In summary, miR-21, under normoxic conditions, was overexpressed in the group of neurospheres submitted to ionizing radiation treatment. This suggests that its role in the regulation of HIF-1α is related to radioresistant neurospheres. miR-326, under normoxic conditions, was associated with an increased risk of recurrence in both groups, also demonstrating that this positive regulation does not depend on ionizing radiation treatment.

Conflict of interest
The authors declare that they have no conflict of interest.

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