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Original Paper

Downregulation of MicroRNA-330 Correlates with the Radiation Sensitivity and Prognosis of Patients with Brain Metastasis from Lung Cancer

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Key Words
MicroRNA-330 • Lung cancer with brain metastasis • Radiation therapy • Sensitivity • Prognosis • Follow-up

Abstract
Background: The present study sought to explore the role of microRNA-330 (miR-330) in predicting the radiation response and prognosis of patients with brain metastasis (BM) from lung cancer (LC). ***Methods:*** Patients with BM from LC were identified and classified into radiation-sensitive and radiation-resistant groups according to the overall survival rate, local and distant recurrence rate after conventional whole-brain radiation therapy. Quantitative real-time polymerase chain reaction (qRT-PCR) was used to detect miR-330 expression in serum. Receiver operating characteristic (ROC) curves were used to evaluate the prognostic value of miR-330 for the radiation sensitivity of brain metastasis from LC. Related clinical factors for radiation sensitivity were assessed by logistic regression analysis, and a survival analysis was conducted using COX regression and the Kaplan-Meier method. ***Results:*** MiR-330 exhibited lower expression in the radiation-sensitive group than in the radiation-resistant group. The area under the ROC curve of miR-330 for predicting radiation sensitivity was 0.898 (optimal cut-off value, 0.815), with a sensitivity of 71.7% and a specificity of 90.1%. After radiation therapy, patients with low miR-330 expression, compared to patients with high miR-330 expression, displayed a lower survival rate and a median survival time. MiR-330 expression was correlated with extracranial metastasis, maximum BM diameter, tumor-node-metastasis (TNM) stage and node (N) stage. Logistic regression and COX regression analyses revealed that extracranial metastasis, TNM stage, N stage and miR-330 expression were factors that influenced both radiation sensitivity and individual prognostic factors in patients with BM from LC. ***Conclusions:*** These findings indicate that the downregulation of miR-330 correlates with radiation sensitivity and poor prognosis in patients with BM from LC.

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Introduction

Lung cancer (LC), including small-cell lung cancer (SCLC) and non-small-cell lung cancer (NSCLC), is the most common cause of cancer deaths worldwide [1-3]. Over 80% of LCs are NSCLC, which confers a very low 5-year survival rate [4]. Currently, radiation therapy has been a common treatment for patients with LC [5-7]. Major causes contributing to poor outcomes in patients with LC include innate or acquired resistance to radiotherapy and high metastatic potential [8, 9]. Invasion and metastasis of cancer cells are critical factors that influence the long-term survival of LC and characterized by the capability to invade to adjacent area, extravagate into lymphatic and blood vessels and colonize into a distance environment [10]. Brain metastasis (BM) is one of the direst complications of LC [11] and is a crucial prognostic factor for LC [12]. BM can cause significant cognitive, neurologic and emotional difficulties and can negatively impact patient survival [13], with a median overall survival of 4.5 months for patients receiving standard whole-brain radiation therapy after they were diagnosed with BM in NSCLC [14]. The accurate assessment of BM plays a critical role in LC management, and an early diagnosis can result in a better survival [15]. Previous efforts to characterize patients with LC who will develop BM have been disappointing [13]; thus, we resort to molecular mechanisms in order to provide a more accurate evaluation of patients with BM from LC. MicroRNAs (miRNAs) are non-coding RNAs of approximately 18-24 nucleotides that can bind to the sequences of target mRNAs in the 3'-untranslated region (3'-UTR) and modulate gene expression at the post-transcriptional level [16, 17], therefore affecting a wide variety of biological processes, including proliferation, differentiation as well as and apoptosis. Thus, these miRNAs are associated with a diverse array of human diseases, including cancer [18-20]. The altered expression of miRNAs has been commonly found in LC [21, 22], and accumulatively been evidenced to involve in carcinogenesis and tumor progression in LC [23-25]. As a member of the miRNA family, microRNA-330 (miR-330) has two subsidiary strands, miR-330-3p and miR-330-5p [26]. Serum miR-330 is reported to function as a tumor suppressor in prostate cancer [27, 28]. Evidence also shows that the elevated expression of miR-330 can suppress the proliferation of colorectal cancer cells *in vivo* [29]. However, the functions and molecular mechanisms concerning miR-330 in the regulation of LC are currently unknown. Moreover, serum miRNAs can function as novel biomarkers to predict the response to radiotherapy and the prognosis clinically [30, 31]. By using quantitative real-time polymerase chain reaction (qRT-PCR) to detect miR-330 expression in serum, our study aimed to investigate the role of miR-330 in predicting the radiation sensitivity and prognosis of patients with BM from LC.

Materials and Methods

Study Subjects

A total of 258 patients with LC and brain metastasis diagnosed at the Second Affiliated Hospital of Jinzhou Medical University from January 2010 to December 2015 were identified. All subjects (153 males and 105 females) had a median age of 59 years, ranging from 35 to 75 years. The subjects were confirmed based on cytology or histopathology, and brain metastasis was confirmed by surgical pathology or clinical diagnosis, such as physical signs, brain computed tomography, clinical symptoms and nuclear magnetic resonance. The inclusion criteria were patients with complete medical records, diagnosis of the primary tumor as brain metastasis, patients receiving no surgical treatment but whole-brain radiation therapy, no acute hemorrhage or abnormal routine blood work, at least 1 assessable tumor lesion or measurable intracranial metastatic lesion by magnetic resonance imaging and a Karnofsky Performance Scale (KPS) score no less than 60 points [32]. Exclusion criteria were patients with mental abnormalities; patients with severe dysfunction in the heart, liver, kidney and other organs; and patients with other tumor lesions except for brain metastatic lesions and primary tumors. The general information and clinical records of all subjects are listed in Table 1. Informed consent was obtained from all subjects, and the investigation was approved by the ethical committee of the Second Affiliated Hospital of Jinzhou Medical University.

Whole-Brain Radiation Therapy

All subjects received conventional whole-brain radiation therapy with a Nucletron-Simulix simulator (Nucletron B.V., Veenendaal, The Netherlands) [33]. The patients put in the supine position, with the head fixed by a U-shape thermoplastic mask and supported by a pillow. The front and back of the head and the upper bound were exposed. The lower bound was along the supraorbital, orbital, front and bottom of the sphenoid sinus; the top of the nasopharynx; and the inferior border of the C1 spine. The radiation therapy dose was 30 Gy (3 Gy × 10 fractions) for 5 weeks. The patients were classified into the radiation-sensitive group and the radiation-resistant group based on the overall survival rate and the distant and local recurrence rate [34].

Table 1. General information and clinical characteristics of patients. BM, brain metastasis; TNM, tumor-node-metastasis; N, Node

Characteristics	Category	Case
Gender	Male	153
	Female	105
Age (Year)	≤ 60	146
	> 60	112
Pathological type	Adenocarcinoma	149
	Squamous cell carcinoma	61
	Other types	48
Extracranial metastasis	Without	142
	With	116
Number of brain metastatic lesions	1	94
	≥ 2	164
Maximum diameter of BM (cm)	≤ 2	95
	> 2	163
TNM stage	T1	142
	T2	85
	T3	31
N stage	N0	29
	N1	58
	N2	171

Table 2. Primer sequences of the target genes. F, Forward; R, Reverse; miR-330, microRNA-330

Target gene	Primer sequence
miR-330	F: 5'-GGGCTCGAGCCACTCACCCACACTGAAGA-3'
	R: 5'-GGGGCGGCCGCGTTTCTCCCTCTGCTTGACG-3'
U6	F: 5'-CGCTTCGGCAGCACATATACTA-3'
	R: 5'-CGCTTCACGAATTTGAGTGTCA-3'

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Venous blood (10 mL) was obtained from fasting patients in the morning before radiation therapy, and serum was collected after centrifugation. Total RNA was extracted using a Trizol total RNA Extraction Kit (Shanghai Sangon Biological Engineering Technology & Services Co., Ltd., Shanghai, China) and was then reverse transcribed into complementary DNA (cDNA). Total RNA (1 μL), 50 μmol/L Oligo-dT Prime (0.5 μL), 5 × PrimeScript TM Buffer (2 μL), PrimeScriptTM RT Enzyme (0.5 μL) and 50 μmol/L Random 6-mers (0.5 μL) were obtained with the addition of RNase-free dH_2O to a total volume of 20 μL. The solution was mixed and reacted at 37°C for 15 min and then at 83°C for 3 s. The reaction was terminated by cooling on the ice, followed by the addition of sterile water (80 μL) and storage at -20°C. Based on the cDNA template, miR-330 expression was detected by a fluorescence quantitative PCR reaction according to the fluorescence quantitative PCR kit (Takara Biotechnology Ltd., Dalian, China). Primer sequences are shown in Table 2. Each group had 3 duplicate wells. The reaction contained Synergy Brands (SYBR) Premix ExTaqTM (2×) (10 μL), 10 μmol/L PCR Primer (0.4 μL), ROX Reference Dye II (50×) (0.4 μL) and cDNA template (2.0 μL), with the addition of dH_2O (6.8 μL) to a total volume of 20 μL. A LightCycler 480 system was used for a fluorescence quantitative PCR reaction with the following reaction conditions: pre-denaturation at 95°C for 10 min, denaturation at 94°C for 15 s, annealing at 60°C for 1 min, and extension at 72°C for 1 min, with 40 cycles. With U6 as an internal reference, the relative mRNA expression was calculated by the $2^{-\Delta\Delta CT}$ method [35].

Follow-up

All patients received follow-up through outpatient visits and by telephone through June 2016, with no cases lost to follow-up. The survival time was assessed monthly, and the diagnosis date was set as the beginning of follow-up. The period from the diagnosis date to death caused by brain metastasis or recurrence was considered the survival time. The totally lost cases, the non-tumor derived deaths and the living patients at the end of follow-up were counted as censored data in conformity with the requirements of statistics.

Statistical Analysis

All data were processed with the Statistical Package for the Social Sciences (SPSS) version 21.0 (SPSS Inc., Chicago, IL, USA). Measurement data are displayed as the mean ± standard deviation (SD). Comparisons between two groups were analyzed by *t* test. Categorical data were detected by the chi-square test, and the rank sum test was used for ranked data. Receiver operating characteristic (ROC) curves were used to evaluate the prognostic value of miR-330 for the radiation sensitivity of brain metastasis from LC. Related clinical factors for radiation sensitivity were assessed by logistic regression analysis, and survival analysis was assessed by COX regression and Kaplan-Meier method. $P < 0.05$ was considered statistically significant.

Results

Clinical Characteristics of the Patients in the Radiation-Sensitive and -Resistant Groups

There were 131 cases in the radiation-sensitive group, including 75 males and 56 females, and 127 cases in the radiation-resistant group, including 78 males and 49 females. Patients in both groups exhibited no significant differences regarding gender, age, pathological type, extracranial metastasis, brain metastasis number, brain metastasis maximum diameter, tumor-node-metastasis (TNM) stage and node (N) stage (all $P > 0.05$) (Table 3).

The miR-330 Expression in Serum in the Radiation-Sensitive and Radiation-Resistant Groups

The results of qRT-PCR indicated that the miR-330 expression in the serum was reduced in the radiation-sensitive group compared to that in the radiation-resistant group ($P < 0.05$, Fig. 1A). ROC curves were applied to evaluate the prognostic value of miR-330 expression for radiation sensitivity. The area under the curve was 0.898, the 95%CI was 0.862~0.934, the optimal cut-off value was 0.815, the sensitivity was 71.7%, and the specificity was 90.1% (Fig. 1B).

Correlation between miR-330 Expression and Clinical Characteristics of Patients with BM from LC

With the optimal cut-off value of 0.815 as a standard for radiation sensitivity, 104 patients had high expression of miR-330 (≥ 0.815), and 154 patients had low expression of miR-330 (< 0.815). The findings implied that miR-330 expression presented no correlation with gender, age, and pathological type, as well as the number and maximum diameter of brain metastases (all $P > 0.05$), while miR-330 expression was correlated with extracranial

Table 3. Clinical characteristics of the patients in the radiation-sensitive and radiation-resistant groups. BM, brain metastasis; TNM, tumor-node-metastasis; N, node

Characteristics	Category	Radiation-sensitive group (n = 131)	Radiation-resistant group (n = 127)	*P*
Gender	Male	75 (49.02)	78 (50.98)	0.528
	Female	56 (53.33)	49 (46.67)	
Age (Year)	≤ 60	73 (50.00)	73 (50.00)	0.611
	> 60	58 (51.79)	54 (48.21)	
Pathological type	Adenocarcinoma	78 (52.35)	71 (47.65)	0.496
	Squamous cell carcinoma	27 (44.26)	34 (55.74)	
	Other types	26 (54.17)	22 (45.83)	
Extracranial metastasis	Without	80 (56.33)	62 (43.67)	0.060
	With	51 (43.97)	65 (56.03)	
Number of brain metastatic lesions	1	46 (48.94)	48 (51.06)	0.699
	≥ 2	85 (51.83)	79 (48.17)	
Maximum diameter of BM	≤ 2 cm	50 (52.63)	45 (47.37)	0.699
	> 2 cm	81 (49.69)	82 (50.31)	
TNM stage	T1	76 (53.52)	66 (46.48)	0.090
	T2	45 (52.94)	40 (47.06)	
	T3	10 (32.26)	21 (67.74)	
N stage	N0	14 (48.28)	15 (51.72)	0.222
	N1	24 (41.38)	34 (58.62)	
	N2	93 (54.39)	78 (45.61)	

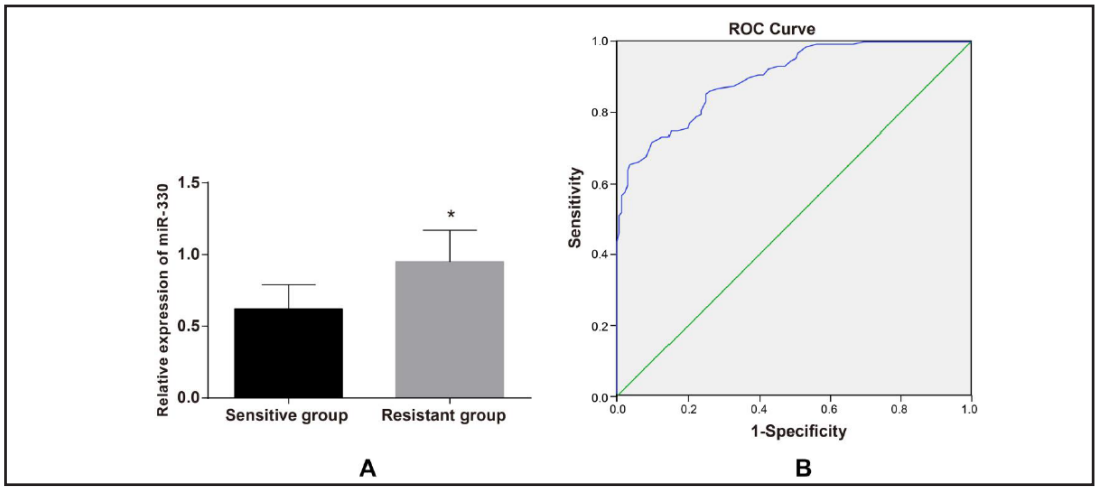

Fig. 1. MiR-330 expression in the serum of the radiation-sensitive and radiation-resistant groups, and the prognostic value for the radiation sensitivity of patients with BM from LC. (A) miR-330 expression in the serum of the radiation-sensitive and radiation-resistant groups; *, $P < 0.05$ compared with the radiation-sensitive group; (B) ROC curve for miR-330 expression in predicting the radiation sensitivity of patients with BM from LC; miR-330, microRNA-330; LC, lung cancer; ROC, receiver operating characteristic; BM, brain metastasis.

Table 4. Correlation between miR-330 expression and the clinical characteristics of patients with BM from LC. LC, lung cancer; BM, brain metastasis; TNM, tumor-node-metastasis; N, node; miR-330, microRNA-330

Characteristics	Category	Case	miR-330		*P*
			Low expression (n = 154)	High expression (n = 104)	
Gender	Male	153	95 (62.09)	58 (37.91)	0.368
	Female	105	59 (56.19)	46 (43.81)	
Age (Year)	≤ 60	146	91 (62.33)	55 (37.67)	0.371
	> 60	112	63 (56.25)	49 (43.75)	
Pathological type	Adenocarcinoma	149	96 (64.43)	53 (35.57)	0.164
	Squamous cell carcinoma	61	31 (50.82)	30 (49.18)	
	Other types	48	27 (56.25)	21 (43.75)	
Extracranial metastasis	Without	142	95 (66.90)	47 (33.09)	0.011
	With	116	59 (50.86)	57 (49.14)	
The number of BM	1	94	62 (65.96)	32 (34.04)	0.147
	≥ 2	164	92 (56.09)	72 (43.91)	
Maximum diameter of BM	≤ 2 cm	95	55 (57.89)	40 (42.11)	0.694
	> 2 cm	163	99 (60.74)	64 (39.26)	
TNM stage	T1	142	90 (63.38)	52 (36.62)	0.004
	T2	85	54 (63.53)	31 (36.47)	
	T3	31	10 (32.26)	21 (67.74)	
N stage	N0	29	19 (65.52)	10 (34.48)	0.019
	N1	58	43 (75.44)	15 (24.56)	
	N2	171	92 (53.49)	79 (46.51)	

metastasis, TNM stage and N stage (all $P < 0.05$). Patients with no extracranial metastasis, high TNM stage and high N stage displayed high miR-330 expression (Table 4).

Logistic Analysis of Factors Influencing the Radiation Sensitivity of Patients with BM from LC

A logistic regression analysis was performed with the radiation sensitivity of patients as a variable and extracranial metastasis, TNM stage, N stage and miR-330 expression as invariables. The results indicated that extracranial metastasis, TNM stage, N stage and miR-330 expression were factors that influenced the radiation sensitivity of patients with BM from LC (all $P < 0.05$, Table 5).

Correlation between miR-330 Expression and Survival Time of Patients with BM from LC

At the 6th month and the 1st year after radiation therapy, the survival rates of patients with low miR-330 expression were 86.36% and 75.97%, respectively, and the median survival

Table 5. Logistic regression analysis of the factors that influence the radiation sensitivity of patients with BM from LC. LC, lung cancer; BM, brain metastasis; TNM, tumor-node-metastasis; N, node; miR-330, microRNA-330; SE, standard error; Exp., exponential

Factor	B	SE	Wald	*P*	Exp. (B)	95.0%CI for Exp. (B)
Extracranial metastasis	1.102	0.318	12.026	0.001	3.010	1.615~5.612
TNM stage	0.461	0.231	3.980	0.046	1.585	1.008~2.493
N stage	0.409	0.208	3.858	0.049	1.505	1.001~2.262
miR-330	1.040	0.750	13.504	< 0.001	2.830	1.625~4.928

Table 6. Multivariate analysis of clinicopathological features and miR-330 expression and prognosis for patients with BM from LC. LC, lung cancer; BM, brain metastasis; TNM, tumor-node-metastasis; N, node; miR-330, microRNA-330; SE, standard error; Exp., exponential; df, degree of freedom

Factor	B	SE	Wald	df	*P*	Exp. (B)	95.0%CI for Exp. (B)
Extracranial metastasis	0.525	0.203	6.682	1	0.010	1.691	1.135~2.518
TNM stage	0.144	0.138	4.746	1	0.029	1.155	1.015~1.315
N stage	0.801	0.132	11.990	1	0.001	2.228	1.416~3.507
miR-330	0.706	0.198	14.519	1	< 0.001	2.027	1.409~2.915

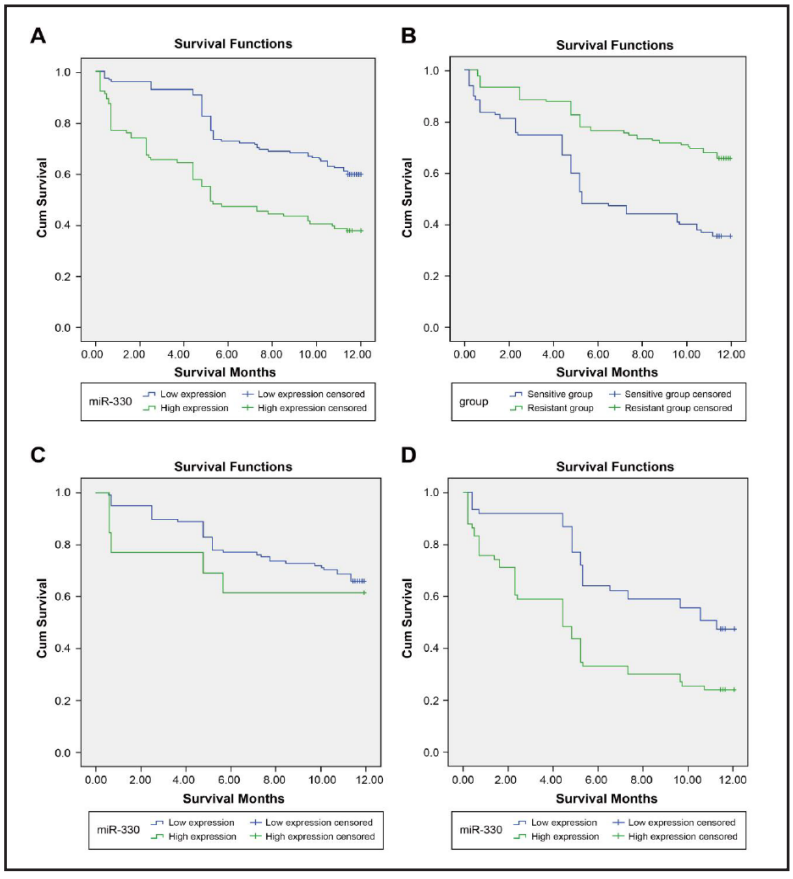

Fig. 2. Kaplan-Meier survival curves for patients with BM from LC. (A) Kaplan-Meier survival curves for patients with LC who have high and low miR-330 expression levels; (B) Kaplan-Meier survival curves for patients with LC in the radiation-sensitive and radiation-resistant groups; (C) Kaplan-Meier survival curves for patients with LC who have high and low miR-330 expression levels in the resistant group; (D) Kaplan-Meier survival curves for patients with LC who have high and low miR-330 expression levels in the sensitive group; miR-330, microRNA-330; LC, lung cancer; BM, brain metastasis.

time was 9.5 months, which is significantly higher than the corresponding survival rates (26.92%, 21.15%) and median survival time (6.6 months) of patients with high miR-330 expression. The corresponding survival rates and the median survival time in the radiation-sensitive group were 77.09%, 69.47% and 9.7 months, which are significantly higher than those in the radiation-resistant group (47.24%, 37.80% and 7.2 months). In the radiation-sensitive group, the patients with low miR-330 expression showed higher survival rates (85.59% and 77.12%) and a higher median survival time (9.7 months) than the patients with high miR-330 expression (27.78%, 0% and 8.3 months). In the radiation-resistant group, the patients with low miR-330 expression showed higher survival rates (88.89% and

72.22%) and a higher median survival time (8.7 months) to the patients with high miR-330 expression (60.44%, 52.75% and 5.4 months) (all $P < 0.05$, Fig. 2).

Multivariate Analysis of Clinicopathological Features and miR-330 Expression and Prognosis for Patients with BM from LC

After quantitative assignment to extracranial metastasis, tumor diameter, TNM stage, N stage, and miR-330 expression, the COX model was established with $P < 0.05$ as a significant standard for variables. The results of multivariate analysis demonstrated that extracranial metastasis, TNM stage, N stage and miR-330 expression were individual risk factors for the prognosis of patients with BM from LC (Table 6).

Discussion

Various researchers have identified miRNAs as potential biomarkers for the diagnosis, prognosis and prediction of the response to radiation therapy in tumorigenesis [30, 36, 37]. Promising roles of miRNA in carcinogenesis have been shown to be closely related to the epithelial-mesenchymal transition, cancer stem cell properties, tumor metastasis initiation and response to radiotherapy or chemotherapy [38]. The altered expression of miR-330 has been found in the carcinogenesis of various tumors [39, 40]. The present study sought to investigate miR-330 expression in patients with BM from LC following conventional whole-brain radiation and the role of miR-330 in radiation sensitivity and prognosis. Our research provided evidence that miR-330 expression was downregulated in radiation-sensitive patients with BM from LC and was associated with a lower survival rate and median survival time. Therefore, miR-330 can function as a potential prognostic biomarker and predict radiation sensitivity in patients with BM from LC.

The findings of our study revealed that miR-330 exhibited lower expression in the radiation-sensitive group than in the radiation-resistant group. Current studies concerning miR-330 have mostly concentrated on its role in various cancer types by regulating Sp1, CDC42 and E2F1. MiR-330 can function as a tumor suppressor by inhibiting the translation of Sp1 and E2F1 in prostate cancer, and reduced E2F1 protein expression exerts a downstream effect of downregulating the levels of p-protein kinase B (Akt) and initiating pro-apoptotic pathways, while the downregulation of the Sp1 protein displays an anti-metastatic effect [27, 41]. A previous study suggested that the E2F1/p-Akt pathway improves cell survival in response to cytotoxic insult caused by chemotherapeutics and radiation, and miR-330-3p expression is inversely correlated with the expression of the E2F1 protein [42]. In colorectal cancer, it has also been reported that miR-330 can serve as a tumor suppressor by suppressing CDC42 translation and negatively regulating the proliferation of colorectal cancer cells [29]. Regarding miR-330, it remains unknown which strand of the miRNA duplex, miR-330-3p or miR-330-5p, performs as the functionally mature strand [26]. MiR-330-3p can control cell proliferation by targeting the early growth response 2 in NSCLC [4]. Indeed, miR-330-5p is also identified to be a credible binding site in the E2F1 mRNA sequence [43]. Yoo et al. reported that downregulated miR-330-5p expression can affect colorectal cancer development [40]. Bibby and colleagues observed an increase in radioresistance due to miR-330-5p silencing, suggesting that the downregulation of miR-330-5p in tumors can alter the response to radiotherapy in esophageal adenocarcinoma [42], which is consistent with our results.

It was also found that at the 6th month and the 1st year after whole-brain radiation therapy, patients with low miR-330 expression displayed a lower survival rate and median survival time than patients with high miR-330 expression, indicating that the downregulation of miR-330 expression was associated with a lower survival rate and median survival time. MiRNAs are often found to be related to the survival and prognosis of patients with different cancers [44-47]. Zhang et al. demonstrated that miR-221 overexpression is correlated with poor prognosis in patients with NSCLC [48]. MiR-148b expression was also shown by Ge et

al. to be independently related to the overall survival of patients with NSCLC [49]. Kim et al. also indicate that the expressions of miR-200c and miR-126 are associated with prognosis in patients with NSCLC [50]. Deng et al clarified the metastatic-inhibitory effect of miR-193a-3p on LC cells by deregulating the expression of tumor-related proteins [9]. It is safe for us to speculate on the prognostic value of miR-330 as a suppressor in cancers. Furthermore, miR-330 was correlated with extracranial metastasis, maximum BM diameter, TNM stage and N stage. Logistic regression and Cox regression analyses revealed that extracranial metastasis, TNM stage, N stage and miR-330 expression were factors that influence radiation sensitivity and were individual prognostic factors for patients with BM from LC. MiRNA-200c and ETAR mRNA expressions have been revealed to be associated with TNM stage and N stage in patients with advanced NSCLC [51]. Additionally, miR-197 and miR-184 are reported to be correlated with BM from LC [52, 53].

To conclude, our data revealed that miR-330 was expressed at low levels in radiation-sensitive patients with BM from LC and was associated with a lower survival rate and a median survival time. These results suggested that the downregulation of miR-330 expression was sensitive to radiation therapy and correlated with a poor prognosis in patients with BM from LC. Therefore, miR-330 may represent a novel predictor for radiation sensitivity and a therapeutic target for the prognosis of patients with BM from LC. Further studies based on different radiation doses for the patients with BM from LC will be carried out in the future to confirm and develop our conclusions.

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Disclosure Statement

The authors declare no conflict of interest.

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