miR-10a and miR-204 as a Potential Prognostic Indicator in Low-Grade Gliomas

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Ju Cheol Son[1], Hyoung Oh Jeong[1], Deaui Park[2,3], Sang Gyoon No[1], Eun Kyeong Lee[3], Jaewon Lee[1,4] and Hae Young Chung[1,4]

[1]Interdisciplinary Research Program of Bioinformatics and Longevity Science, Pusan National University, Busan, Republic of Korea. [2]Department of Predictive Toxicology, Korea Institute of Toxicology, Daejeon, Republic of Korea. [3]Center for Convergent Research of Emerging Virus Infection, Koera Research Institute of Chemical Technology, Deajeon, Republic of Korea. [4]Molecular Inflammation Research Center for Aging Intervention (MRCA), College of Pharmacy, Pusan National University, Busan, Republic of Korea.

ABSTRACT: This study aimed to identify and characterize microRNAs (miRNAs) that are related to radiosensitivity in low-grade gliomas (LGGs). The miRNA expression levels in radiosensitive and radioresistant LGGs were compared using The Cancer Genome Atlas database, and differentially expressed miRNAs were identified using the EBSeq package. The miRNA target genes were predicted using Web databases. Fifteen miRNAs were differentially expressed between the groups, with miR-10a and miR-204 being related to overall survival (OS) of patients with LGG. Patients with upregulated miR-10a expression had a higher mortality rate and shorter OS time, whereas patients with downregulated miR-204 expression had a lower mortality rate and longer OS time. Two genes, *HSP90AA1* and *CREB5*, were targets for both miRNAs. Thus, this study suggests that expression of miR-10a and miR-204 is significantly related to both radiosensitivity and the survival of patients with LGG. These miRNAs could therefore act as clinical biomarkers for LGG prognosis and diagnosis.

KEYWORDS: miRNA, low-grade glioma, overall survival (OS), radiosensitivity, HSP90AA1, CREB5

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CORRESPONDING AUTHOR: Hae Young Chung, Molecular Inflammation Research Center for Aging Intervention (MRCA), College of Pharmacy, Pusan National University, Busandaehak-ro 63beon-gil, Geumjeong-gu, Busan 46241, Republic of Korea.
Email: hyjung@pusan.ac.kr

Introduction

Glioma is a type of primary brain neoplasm derived from neuroglial cells. Low-grade gliomas (LGGs), classified as grade 1 or 2 by the World Health Organization,[1] account for approximately 15% of all glial tumors. Patients with LGG have a median survival of more than 10 years, depending on prognostic factors such as tumor status and patient health. Although there is debate surrounding what comprises the optimal treatment regime for LGG, surgical resection or biopsy followed by radiotherapy (RT) is the most widely used protocol. Recent advances in RT procedures, such as the use of CyberKnife and Gamma Knife technology in radiosurgery, mean that malignant tissue can be effectively removed with little impact on normal brain tissues. Indeed, prospective randomized clinical trials featuring adult patients with supratentorial LGGs showed improved survival rates when an early adjuvant RT procedure was used.[2] However, gliomas are known for recurrence, which is mainly caused by chemoresistance and radioresistance. Significant component of gliomas resistance is signaling pathways involved in the maintenance of stem cell–like phenotype.[3]

MicroRNAs (miRNAs) are conserved non–protein-coding RNAs related to negative regulation of gene expression by one or more messenger RNAs with imperfect base complementarity.[4] The negative regulatory function of miRNAs can alter multiple biological processes, including apoptosis, cellular proliferation, tumorigenesis, drug resistance, and response of cells to radiation.[5] Several reports have described miRNAs as key regulators of different pathways related to development of gliomas and their role in therapeutic resistance.[6] High-throughput methods have been used to discover radiation-related miRNAs, and alterations in the levels of these miRNAs have been shown to modulate the radiosensitivity of several malignances, including glioblastoma,[7] laryngeal carcinoma,[8] and lung cancer.[9] However, the relevance of miRNAs to the radiosensitivity of human LGGs remains unclear.

With the progression of high-throughput genomic technologies, several studies have revealed that bioinformatics approaches have been applied to determine the biological basis of malignant transformation of LGGs.[10,11] However, to our knowledge, there are no papers that describe the genetic comparison of tumor tissues from radiosensitive and radioresistant LGG subjects to discover genetic biomarkers. In this study, we evaluated the global expression of miRNAs in LGG tumors from patients undergoing primary radiation therapy. We then assessed the relationship between miRNA expression and patient survival to identify whether the radiosensitivity-related miRNAs affect the patient's survival.

Materials and Methods

miRNA expression data sets

MicroRNA expression data from LGG patient samples were obtained from The Cancer Genome Atlas (TCGA) database (http://gdc-portal.nci.nih.gov). The selection criteria for the patient data sets within this database were the word "No" in

the "History of neoadjuvant treatment" field and the phrase "Primary tumor field" in the "Anatomic treatment site" field. Data sets from patients with a history of chemotherapy (CT) were excluded. The follow-up information for most of the patients was incomplete, and so we selected data sets from LGG patients with the most information in the fields "Primary therapy outcome success" and "Person neoplasm cancer status." In total, miRNA expression profiles from 10 patients with radioresistant LGGs and 24 patients with radiosensitive LGGs remained after applying the filtering steps described above (Supplementary Table 1). Where there was a logical conflict between the contents of the "Primary therapy outcome success" and "Person neoplasm cancer status" fields, for instance, "with tumor" and "complete response," the data set was excluded.

Identification of radiosensitivity-related miRNA expression level

We used level 3 miRNA expression data sets of LGGs processed by the Broad Institute's TCGA workgroup. These level 3 data comprise reads per kilobase per million mapped reads (RPKM), which are raw counts of gene expression derived from the Illumina HiSeq sequencing results. Using the EBSeq program of the R package, we calculated radiosensitivity-related differentially expressed miRNAs in the RT-sensitive and RT-resistant groups compared with 135 samples (control) which have no RT or CT history. Normalized expression levels for these samples were further examined for each investigated miRNA. Significant differential expression of miRNAs were defined by a |log fold change| of >1.0 and a posterior probability of differential expression (PPDE) threshold of 0.95.

Clustering analysis

Clusters of data sets showing similar expression profiles were examined by clustering analysis using Hierarchical Clustering (HCL) package from the MultiExperiment Viewer (MeV) software application. Median RPKM data were normalized using the *Normalized Gens/Rows* function of MeV software. The significance of similarities was assessed with Pearson correlation analysis.

Survival analysis

Kaplan-Meier analysis was conducted to examine the overall survival (OS) of the selected LGG samples (n = 464), and the significance of the discriminations between high and low expression levels of selected miRNAs was assessed using the log-rank test. The OS was defined as the time from the date of diagnosis to the final follow-up date. A statistically significant difference between groups was defined by a P value of <.05.

Prediction of radiosensitivity-related miRNA target genes

To predict targets of the differentially expressed miRNAs, 2 online miRNA databases were merged: miRTarBase[12] and miRecords.[13] miRTarBase provides information about experimentally validated miRNA-target interactions, but miRecords contains predicted miRNA targets produced by 11 established prediction programs and validated miRNA targets resulting from meticulous literature. In this study, we used only validated miRNA-target interactions.

Results

Grouping of patients with LGG

To examine whether the treatment method affected miRNA expression in LGG tumors, patients with LGG whose tumor miRNA had been sequenced were selected from the TCGA database. Of the 530 open cases in the database, 188 patients were monitored primarily by clinical follow-up. Using the treatment outcomes defined in the database, cases were categorized as either sensitive ("complete remission/response") or resistant ("stable disease," "progressive disease," or "partial remission/response"). Cases were then further categorized according to the treatment method, which was RT, CT, or combination therapy (CT + RT). The categorized data are summarized in Table 1. Next, the miRNA expression profiles in the different groups were compared using MeV software, and, as expected, distinct clusters were seen for the RT and CT groups. Interestingly, although the sensitive and resistant tumors within the treatment groups were clearly distinct, the RT and CT + RT groups clustered more closely (Figure 1). Overall, these data suggest that RT had a greater effect on miRNA expression than CT.

Detection of differentially expressed miRNAs

The Bioconductor package EBSeq was conducted to find differentially expressed miRNAs between the RT-sensitive and RT-resistant groups, and the fold change in miRNA expression between the LGG tumors from patients receiving only RT and control patients not receiving RT and CT was calculated. In total, 15 miRNAs met the cutoff criteria of a PPDE >0.95 and a |fold change| of >2.0. Of these, only 2 miRNAs (miR-10a and miR-122) were differentially expressed in the RT-resistant group, whereas the remaining 13 were differentially expressed in the RT-sensitive group. Of these 13, 5 miRNAs (miR-216a, miR-216b, miR-217, miR-4662a, and miR-4703) were significantly upregulated in the treatment group, and the other 8 miRNAs (miR-135b, miR-204, miR-34b, miR-34c, miR-3925, miR-449a, miR-517a, and miR-517b) were significantly downregulated in the treatment group. The fold changes and PPDE for the 15 significantly differentially expressed miRNAs are presented in Table 2.

Table 1. Low-grade glioma data sets used in analyses, obtained from The Cancer Genome Atlas database.

TREATMENT METHOD	DESCRIPTION	NO. OF SAMPLES
Radiotherapy	Sensitive	24
	Resistant	10
Chemotherapy	Sensitive	4
	Resistant	11
Combination therapy	Sensitive	38
	Resistant	101

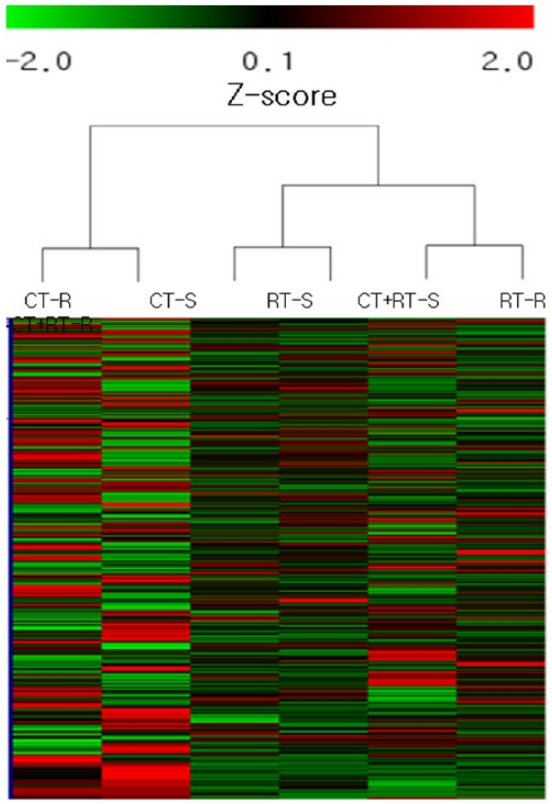

Figure 1. Cluster analysis of the 975 miRNA expression profile of LGGs, classified by treatment. Heat map showing the hierarchical clustering of global miRNA expression data for patients treated with RT (n = 34), CT (n = 15), or combined treatment (CT + RT) (n = 139). Rows represent miRNAs and columns represent sample groups. Red and green bars, respectively, indicate high and low median RPKM values relative to each treatment sample. CT indicates chemotherapy; miRNA, microRNA; R, resistant; RPKM, reads per kilobase per million mapped reads; RT, radiotherapy; S, sensitive.

Survival analysis

To investigate whether the radiosensitivity-related miRNAs affect the patient's life span, the relationship between expression of the 15 miRNAs and OS of patients with LGG was analyzed using Kaplan-Meier survival analysis and the log-rank test. For this analysis, the expression level of each miRNA for each sample was classified as either "high" or "low" using the mean expression level in the control tissues as a cutoff. According to this analysis, the expression levels of miR-10a and miR-204 were significantly correlated with OS in this cohort ($P = 0$ and $P = .005$, respectively; Figure 2A and B), but other miRNAs were not significant (Supplementary Figure 1). Upregulation of miR-10a was related to a high mortality rate and shorter OS, whereas downregulation of miR-204 was associated with a low mortality rate and longer OS. Taken together, these data suggest that the miR-10a and miR-204 play an important in the progression of LGGs.

In silico identification of possible miRNA targets

Having shown that the expression level of the 2 miRNAs described above was significantly related to LGG patient survival, possible miRNA targets, and thus molecular mechanisms that might explain this association, were investigated. Validated targets for the miRNAs were obtained using 2 Web-based databases (miRecords[13] and miRTarBase[12]), and 307 and 72 potential target genes were obtained for miR-10a and miR-204, respectively (Supplementary Table 2).

Discussion

Radiotherapy uses high-energy radiation and different types of radiation, such as gamma rays, charged particles, and heavy ions, to kill cancers, but, despite extensive research efforts, intrinsic and acquired radioresistance remains a significant problem. It could be that miRNAs are associated with the development of therapeutic resistance, and aberrant miRNA expression was implicated in cancer progression[14] as well as radioresistance and chemoresistance in brain tumor.[6] The determination of the functional and clinical importance of specific miRNAs might therefore be useful in therapeutic decision making.

Changes in miRNA expression following RT for the treatment of various cancers, as well as specific roles for several miRNAs in the development of radiosensitivity, have been reported. Consistent with these published findings, 15 miRNAs between the RT-sensitive and RT-resistant groups were observed in this study. Xiao et al[7] revealed that miR-135b, which was decreased in a RT-sensitive group (Table 2), contributed to radioresistance in glioblastoma multiforme (GBM), whereas low levels of miR-135b were more sensitive to radiation and thus more impressionable to death in glioma cells. miR-122, which was downregulated in the RT-resistant group (Table 2), has well-established tumor suppressor activity, although Wang et al[15] found that miR-122 level was inversely associated with the survival time of patients with GBM after resection. The expression level of miR-10a decreased after irradiation in thyroid cancer cells[16] and lymphocytes,[17] but the correlation between miR-10a and miR-204 expression and radiosensitivity is not known.

Kaplan-Meier analysis showed that increased or decreased expression of miR-10a or miR-204, respectively, was significantly associated with LGG patient survival, these being the miRNAs that were differentially regulated in response to RT. Several reports have shown that miR-10a was aberrantly increased in many malignancies,[18,19] and Weiss et al[20] observed increased level of miR-10a in highly metastatic pancreatic cancer. In addition, Yu et al[21] reported that miR-10a, which was remarkably increased in lung cancer, targeted phosphatase and tensin homolog (PTEN) to promote metastasis, and Li et al[22] revealed a relationship between miR-10a level and both disease-free survival and OS in gastric cancer. Although the function of miR-10a in LGG progression has not been previously analyzed, our reports also show that this gene could function as a proto-oncogene in this disease.

Table 2. miRNAs that were differentially expressed between the RT-sensitive and RT-resistant groups.

	mIRNA	FOLD CHANGE	PPDE
RT-R/Con	has-miR-10a	6.45	0.99
	hsa-miR-122	−9.39	0.99
RT-S/Con	hsa-miR-216a	3.11	1
	hsa-miR-216b	4.84	1
	hsa-miR-217	2.12	0.99
	hsa-miR-4662a	2.25	0.99
	hsa-miR-4703	3.28	0.98
	hsa-miR-517a	−2.90	0.99
	hsa-miR-517b	−2.90	0.99
	hsa-miR-135b	−5.07	0.99
	has-miR-204	−3.07	0.97
	hsa-miR-34b	−11.52	0.99
	hsa-miR-34c	−11.25	0.99
	hsa-miR-3925	−22.16	0.99
	hsa-miR-449a	−44.99	0.99

Abbreviations: miRNA, microRNA; PPDE, posterior probability of differential expression; RT, radiotherapy.

miR-204 can reportedly act as a tumor suppressor gene in several malignancies, including gastric cancer,[23] glioma,[24] and head and neck cancer.[25] In addition, Yin et al[26] reported that miR-204-5p can inhibit cell survival and accelerate the impact of CT in colorectal cancer. However, these results were contradicted by Sümbül et al,[27] who reported significantly higher levels of miR-204-5p in colorectal cancer than in a healthy group. In the latter study, it was suggested that the increased expression of miR-204-5p in colorectal cancers acted to overcome cellular autophagy by inhibiting the expression of *LC3B-II* and *Bcl-2*. To date, there are no published reports of the relationship between miR-204 expression and prognosis, OS, and radiotherapeutic response in patients with LGG.

As miRNAs function posttranscriptionally in biological processes, we used a bioinformatics method to discover target genes for miR-10a and miR-204 before analyzing the potential mechanisms by which they could affect LGG patient survival. Considering the literature and the KEGG pathway obtained by mapping the targets, the PI3K-AKT pathway was a potential

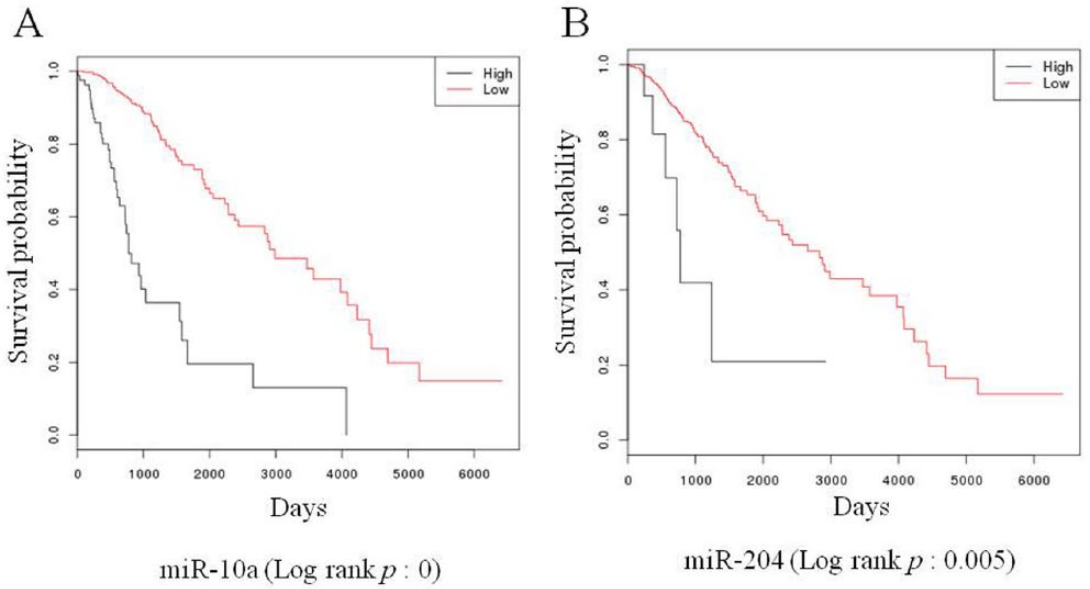

Figure 2. Overall survival of patients with LGG. Kaplan-Meier plots showing the overall survival of patients with LGG with high or low expression of either (A) miR-10a or (B) miR-204. High and low expression level cutoffs were determined using the mean expression level of control patients. LGG indicates low-grade glioma; miR, microRNA.

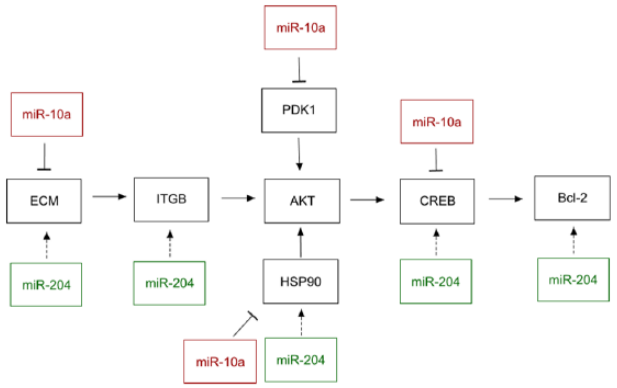

Figure 3. The potential predictive markers based on the KEGG map and literature, showing the involvement of miR-10a and miR-204. Red boxes indicate higher miRNA expression in the RT-resistant group, whereas green boxes indicate lower miRNA expression in the RT-sensitive group. miRNA indicates microRNA; RT, radiotherapy.

predictor that could explain the mechanism of radiosensitivity. Within this pathway, the genes targeted by miR-10a were *HSP90AA1*, *CREB5*, *PDK1*, *and ECM (COL4A2*, *COL4A3*, *COL6A2*), whereas those targeted by miR-204 were *HSP90AA1*, *CREB5*, *Bcl-2*, *ITGB*, and *ECM* (*IBSP*) (Figure 3). *CREB5* and *HSP90AA1* were notified to be modulated by both miR-10a and miR-204. *HSP90AA1* is critical for maintaining the stability of cancer-related proteins, including *AKT*, and *CREB5* is a transcription factor that modulates the expression of genes such as *Bcl-2*. We showed that level of miR-204 was decreased in the RT-sensitive group, and lower levels of miR-204 would be expected to lead to the derepression of *CREB5* and, consequently, higher expression of *Bcl-2*.

The relationship between increased *Bcl-2* expression level and longer survival is in contrast to the well-described anti-apoptotic role of *Bcl-2*, but it is possible that the impact of *Bcl-2* varies according to the context. The expression of *Bcl-2* is related to limited survival in prostate cancer[28] but improved clinical results in several malignant. Poincloux et al[29] found that downregulated *Bcl-2* expression tended to more aggressive tumors and tumor recurrence in colon cancer. Similarly, Baretton et al[30] revealed that higher *Bcl-2* expression was related to better clinical outcomes in colorectal cancer, whereas decreased expression of *Bcl-2* more typically occurred in carcinomas missing detectable *p53* expression, and another study found that *Bcl-2* level was more increased in colorectal adenomas than in invasive tumors.[31] *Bcl-2*-positive cells were found to be consistently more abundant in LGGs than in high-grade gliomas.[32,33] Taken together, tumors expressing a high level of *Bcl-2* are likely to be less invasive and to progress more slowly.

Conclusions

In this study, we have shown that miR-10a and miR-204, differentially expressed 2 miRNAs between RT-sensitive and RT-resistant LGGs, are closely correlated with OS of patients with LGG. These observations could potentially be explained by alterations in the expression of *HSP90AA1* and *CREB5, 2* genes that are regulated by both miRNAs, and consequent alterations in *Bcl-2* expression. The expression level of miR-10a and miR-204 could potentially be used as a prognostic indicator for patients with LGG.

Author Contributions

HOJ, JL and HYC conceived and designed the experiments. HOJ and EKL wrote the first draft of the manuscript. SGN and DP contributed to the acquisition of the results and to formulating conclusions. All the authors were involved equally in the development of the article's discussion points and structure. All the authors reviewed and approved the final manuscript.

Disclosures and Ethics

This manuscript was subjected to blind peer review by at least 3 independent experts and was scanned with anti-plagiarism software on submission. All the authors approved the article prior to publication and confirmed their compliance with all applicable ethical and legal requirements, including validity of authorship, disclosure of competing interests and funding sources, the ethical treatment of human and animal study participants, and third-party copyright requirements.

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