MicroRNA expression in pediatric intracranial ependymomas and their potential value for tumor grading

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Abstract. Intracranial ependymoma represents one of the most common pediatric central nervous system malignancies, and exhibits a wide range of clinical behavior from relatively indolent lesions to highly malignant anaplastic ependymomas. Due to the heterogeneous nature of this disease there is lack of prognostic markers, which would reliably predict the outcome of patients. MicroRNAs (miRNAs) have emerged as important molecules in cancer biology during past decade; however, very little is known about their role in ependymomas. The aim of the present study was to evaluate expression of miRNAs in archived formalin-fixed paraffin-embedded (FFPE) samples of pediatric intracranial ependymomas. The expression of miRNAs were examined in 29 samples of ependymoma and we observed that miR-135a-3p, miR-137, miR-17-5p, miR-181d and let-7d-5p were upregulated. In addition, a significantly higher expression of miR-203a was detected in Grade III tumors suggesting its possible use as a prognostic or diagnostic marker. The present study also demonstrated that storage of (FFPE) ependymoma samples for >20 years did not result in a deterioration of miRNAs. The present findings broaden the presently available knowledge regarding miRNA expression in ependymomas and provide further evidence for the employment of miRNA analysis as a supplementary method for the morphological assessment of ependymoma samples.

Introduction

Ependymoma represents one of the most common pediatric intracranial malignancies constituting approximately 9% of all pediatric brain tumors (1,2). Despite their relatively high incidence, there is a lack of appropriate prognostic factors besides extent of surgical resection (3).

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Previous studies have shown that it is hard to predict patient outcome based on histology and/or genetics of the tumor. One of the underlying reason is a heterogeneous nature of the disease (4,5).

The traditional histopathological grading also remains controversial and fails to predict clinical behavior and outcome of the disease (6). The extent of surgical resection still remains the most important predictor of overall survival and time-to-relapse (7). The need for new biomarkers for better diagnosis, grading and management of disease progression is evident.

MicroRNAs are short non-coding molecules that have a major impact on gene expression (8). More than 1,500 miRNAs have been identified in human genome (9). miRNAs have also been shown to play various roles in cancer. Mainly, they have emerged as important regulators in human carcinogenesis by affecting expression of tumor-suppressor genes and oncogenes (10,11).

Although our understanding of the role of miRNAs has substantially improved in recent years, not much attention has been given to the role of miRNAs in ependymomas (12,13). The aim of our work is to broaden our current knowledge of miRNAs' role in pediatric intracranial ependymomas and elucidate their possible use as a new biomarkers and prognostic factors.

Materials and methods

Patients and samples. A total of 29 formalin-fixed paraffin-embedded (FFPE) specimens of ependymomas were collected retrospectively from the archives of Department of Pathology and Molecular Medicine, University Hospital Motol, Prague, Czech Republic. FFPE specimens of ependymoma were obtained from patients treated between years 1985-2017. All patients were under 18 years of age with median age of 6 years (Table I). The histological grade was evaluated independently by two observers according to WHO criteria. Our cohort consisted of 14 grade II tumors and 15 grade III tumors. This study was approved by Ethical committee of Second Faculty of Medicine, Charles University and University hospital Motol, Prague, Czech Republic.

FFPE tissue from plexus choroideus and lining of lateral brain ventricles from 5 patients (median age 11 years) who

died from non-brain-related illnesses was used as a control group.

RNA extraction. Total RNA was extracted from FFPE blocks from five to eight 10 μm-thick tissue sections. The RecoverAll™ Total Nucleic Acid Isolation kit for FFPE (Ambion; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to manufacturer's instructions was used. Total RNA quantity and quality was evaluated using a spectrophotometer (Nanodrop ND-1000, Thermo Fisher Scientific, Inc.).

Relative quantification of miRNA expression. Total RNA extracted as described above was converted to cDNA by reverse transcription using miRNA specific TaqMan® primer and TaqMan® MicroRNA Reverse Transcription kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) according to manufacturer's instructions. qPCR was performed using TaqMan® Individual miRNA assays (14) for miR-135a-3p, miR-137, miR-17-5p, miR-181d, miR-203a, let-7d-5p, RNU48 and miR-596 (Applied Biosystems; Thermo Fisher Scientific, Inc.) and TaqMan® Universal PCR Master Mix according to manufacturer's instructions. All qPCR reactions were performed in triplicate on 96-well plate.

Statistical analysis. For all miRNA quantification experiments cycle threshold (Ct) values were normalized against the expression levels of RNU48. ΔCt values and fold changes were calculated using DataAssist Software v. 3.01 (Applied Biosystems; Thermo Fisher Scientific, Inc.). An unpaired Student's t-test was performed to compare the ΔCt values. P-values of fold changes were adjusted using Benjamini-Hochberg false discovery rate method using DataAssist Software. Statistical significance for all experiments was attributed if $P \leq 0.05$.

Results

Concentration and purity of RNA isolated from long-time archived samples is equal with more recent specimens. Subset of samples from our study was archived in (FFPE) blocks for 25-30 years. In order to verify if the oldest samples in our study are suitable for further analysis we assess purity and concentration of those specimens.

Furthermore, we performed individual RQ-qPCR assay for RNU48 to see variation in threshold cycles. Table II shows that neither RNA concentration nor the ratio of absorbance at 260 and 280 nm (A260/280 nm) does significantly differ between samples of different age. Mean value of A260/280 nm is 1.95 (SD 0.02). Also Ct values of RNU48 was not impaired by the age of FFPE blocks. These results indicate that even the oldest FFPE specimens in our study were suitable for further analysis.

miRNAs are differentially expressed in ependymomas. We performed individual assays for group of miRNAs and the results revealed that miR-135a-3p, miR-137, miR-17-5p, miR-181d and let-7d-5p are differentially expressed in ependymomas when compared to control group ($P<0.05$). All of those miRNAs were overexpressed (Fig. 1, Table III). The miR-203 was found to be overexpressed with the highest difference as compared to control group (fold change 46.3), but the result was not statistically significant ($P=0.08$). Our results also indicate that the miR-34c belonging to the tumor suppressor family miR-34 is (15,16) slightly downregulated in ependymoma samples although the difference was not statistically significant (RQ 0.9, $P=0.75$).

Table I. Clinicopathological features of ependymomas included in the present study.

Age	%	Number
<4 years	34.48	10
4-15 years	55.17	16
>15 years	10.34	3
Grade 2[a]	48.28	14
Grade 3[a]	51.72	15

[a]WHO criteria (4,5).

MiR-203a is overexpressed in grade III tumors. Statistical analysis was performed in order to find differences in expression of miRNAs between grade II and grade III ependymomas. We found miR-203a to have higher expression in grade III ependymomas (Fig. 2, Table III) suggesting its possible role in distinguishing more aggressive tumors. Others miRNAs that are differentially expressed in tumor samples when compared to normal controls did not show statistically significant differences between grade II and grade III tumors.

Discussion

Ependymomas exhibit wide range of biological behavior extending from low-grade lesions to highly malignant tumors. Prognostic stratification based solely on morphology or immunohistochemistry remains elusive and brings only poor results in regards to patient outcome.

In this study we aim at studying expression levels of various miRNAs using individual qPCR assays in order to identify new prognostic and/or diagnostic markers of ependymomas. We have chosen miRNAs based on results of our preliminary experiment (data not shown) where more than 20 miRNAs were involved. Out of this preliminary experiment we have chosen seven miRNAs which were showing differential expression between normal and ependymoma samples or at least trend towards significance for this study.

We have identified miR-135a-3p, miR-137, miR-17-5p, miR-181d and let-7d-5p to be differentially expressed in ependymoma samples. To our knowledge this is the first report of overexpression of miR-137 in pediatric intracranial ependymoma. The let-7d has been previously described to act both as a tumor-suppressor and oncogene (17,18). In ependymomas it was previously shown (12) that let-7d correlates with overall survival. However, we have not found significant difference in let-7d expression between grade II and grade III samples. This result underlines the fact that miRNA are pluripotent molecules and their role in tumorigenesis in given tumor type needs to be elucidated by more studies especially in tumors of genetically heterogeneous nature.

Table II. Comparison of RNA quality and quantity between samples of different ages.

	Concentration		A260/280		Ct RNU48	
Sample age	Mean	SD	Mean	SD	Mean	SD
28-30 years	187 ng/μl	164	1.95	0.073	23.89	0.88
2-5 years	171 ng/μl	103	1.97	0.04	22.63	0.43
1-2 years	173 ng/μl	65	1.94	0.04	23.01	0.14

SD, standard deviation; Ct, cycle threshold; RNU48, small nucleolar RNA, C/D Box 48-used for normalization.

Table III. Table presenting the up- vs. downregulation of miRNAs expression in normal vs. disease samples and grade III vs. grade II cohort.

	N vs. Ep		G3 vs. G2	
Assay	Fold change	P-value	Fold change	P-value
miR-135a-3p	4.0	0.01	1.0	0.15
miR-137	13.0	0.01	0.8	0.10
miR-17-5p	3.7	0.01	0.8	0.50
miR-181d	5.6	0.001	0.6	0.08
miR-203a	46.3	0.08	6.6	0.01
34c	0.9	0.75	1.0	0.40
let-7d-5p	3.6	0.01	1.0	0.06

N, control samples; Ep, ependymoma samples; G2, Grade 2; G3, grade 3.

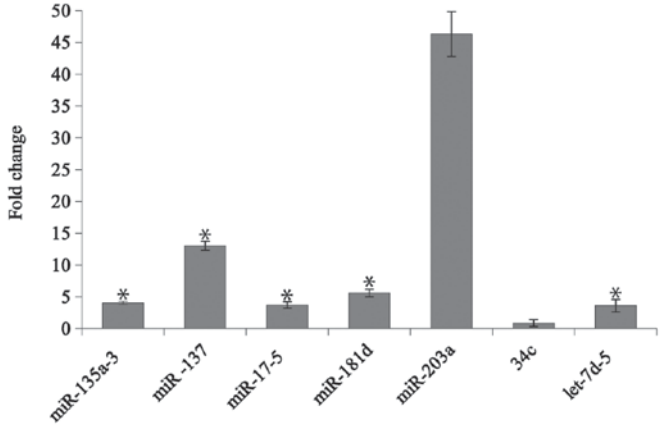

Figure 1. Graph illustrating the expression of different miRNAs in ependymoma samples when compared with a control group, the height of the column thus indicates the number of times the expression in the tumor samples is higher than in control samples. The values in the Y-axis are of fold changes based on DataAssist software. Error bars indicate standard deviation of experiments performed in triplicate. FFPE tissue from plexus choroideus and lining of lateral brain ventricles from 5 patients who died from non-brain-related illnesses were used as a control group. *P<0.05. miRNA, microRNA.

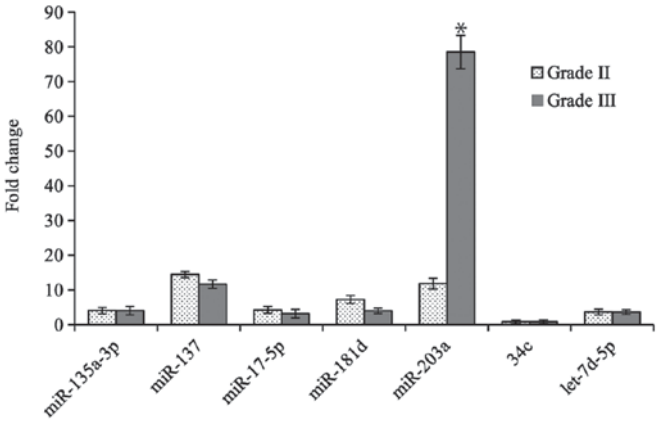

Figure 2. Graph illustrating the expression of different miRNAs in Grade II and Grade III tumors when compared with a control group, the height of the column thus indicates the number of times the expression in the tumor samples is higher than in control samples. The values in the Y-axis are of fold changes based on DataAssist software. Error bars indicate standard deviation of experiments performed in triplicate. FFPE tissue from plexus choroideus and lining of lateral brain ventricles from 5 patients who died from non-brain-related illnesses were used as a control group. *P<0.05. miRNA, microRNA.

Tumor grading in ependymoma reveals high interindividual variability (19). This fact calls for new markers which would make distinguishing between low-grade and high-grade lesion more convenient. We have demonstrated increased expression of miR-203a in grade III tumors compared to grade II tumors. In recent years there has been considerable interest in miR-203. This miRNA located on chromosome 14q32.33 was shown to have aberrant expression in many types of cancers. However, the role of miR-203 in various cancer types is not straightforward. It was previously reported that overexpression of miR-203a in glioblastoma (20) increased apoptosis of cancer cells thus acting as tumor-suppressor gene. Similarly, it was shown that miR-203 upregulation inhibits epithelial-mesenchymal transition in glioblastoma cells (21). miR-203a also suppress hepatocellular carcinoma tumorigenesis by targeting HOXD3 via EGFR-related cell signaling pathways (22). On the other hand, some studies clearly indicated significant correlation between the elevated miR-203 expression and poor overall survival in colorectal adenocarcinoma (23,24) or pancreatic carcinoma (25). miR-203a was also described as a predictor of a poor prognosis in renal cell carcinoma as its silencing inhibits cell proliferation, migration and induces apoptosis (26). Despite relatively common incidence of ependymomas and increased evidence of miRNAs role in various types of cancer, not much attention has been given to role of miRNAs in ependymoma (27). It was shown that miR-17-5p, miR19a-3p

and miR-106b-5p differentiate between grade II and III ependymomas (12). Margolin-Miller *et al* demonstrated that high expression of miR-124-3p in ependymomas significantly correlated with the lower progression-free survival (28).

Our observations of miR-203 expression in ependymomas are in contrast with previous findings (13) which shown that lower expression of miR-203 in ependymoma patients correlated to a trend to develop recurrences. Some evidences suggest that miR-203 might not have sufficient power to predict overall or event-free survival of patient with different types of cancer (29). It was shown that methodology employed in assuming miR-203 expression as well as the ethnicity of a patients might influence the correlation of miR-203 expression and clinical outcome of the patient (24). This could be one of the explanations of discrepancies and needs to be further elucidated.

It was previously shown that PCR amplification results from fresh-frozen material and FFPE samples are similar (30). Routinely archived FFPE samples in pathological departments are therefore suitable for retrospective analysis of miRNAs expression. We have demonstrated that FFPE samples of ependymoma archived for more than 25 years still provide high yields of RNA of excellent purity.

In conclusion, we have shown several miRNAs to be differentially expressed in ependymoma and outlined the role of miR-203. These findings may add to a growing body of literature on our understanding of miRNA expression and their role in ependymoma biology.

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Availability of data and materials

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

Authors' contributions

All authors read and approved the final manuscript. ŠC contributed to evaluation of tumor grade, for RNA extraction, quantification of miRNAs and writing of the manuscript, MB contributed to RNA extraction, quantification of miRNAs, TE contributed to the project design and writing of manuscript, JZ contributed to evaluation of tumor grade, the project design and writing of manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

This study was approved by Ethical committee of Second Faculty of Medicine, Charles University and University hospital Motol, Prague, Czech Republic. Due to the retrospective nature of this study the right to informed consent was waived.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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