Differential expression of microRNA-210 in gliomas of variable cell origin and correlation between increased expression levels and disease progression in astrocytic tumours

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Abstract

Introduction: The microRNA, miR-210, is frequently associated with hypoxia induction, and an increase in its levels is often correlated with poor prognosis in many solid tumours. The present study examines the levels of miR-210 in glioma tumours of multiple origins to determine if an association can be established with disease progression.
Material and methods: Tissue samples were acquired from normal brain tissue, oligodendroglial tumours and astrocytic tumours. The astrocytic tumours were further divided by grade: diffuse astrocytomas (WHO grade II), anaplastic astrocytomas (WHO grade III), and glioblastoma (WHO grade IV). The expression of miR-210 was examined by real-time quantitative RT-PCR. The correlation of the expression of miR-210 and astrocytic tumour grade was analyzed by the Spearman correlation test.
Results: MiR-210 presents a differential expression depending on the origin of the glioma. Oligodendroglial tumours exhibit a significantly reduced level of miR-210 as compared with normal brain tissue. In contrast, astrocytic tumours demonstrate significantly increased levels of miR-210. Furthermore, the expression of miR-210 is positively correlated with the grade of astrocytic tumour, in the following order: grade IV > grade III > grade II > normal brain tissue (p < 0.05).
Conclusions: MiR-210 levels can be potentially established as a biomarker for pathological diagnosis of malignant astrocytic tumour progression. In addition, the expression of miR-210 can be utilized as an additional identification measure of glioma tumour origin.

Key words: astrocytic tumour, oligodendroglial tumour, miR-210, glioma, real-time quantitative RT-PCR.

Introduction

Gliomas are tumours that start in the brain or spine and arise from glial cells. They are characterized by the cell type (ependymal cells, astrocytes, oligodendrocytes, or mixed cells), grade (I-IV), and location (cerebrum or cerebellum). Malignant gliomas account for approximately 70% of the 22 500 malignant primary brain tumours diagnosed each year. Of the malignant gliomas, 60-70% are glioblastomas [37]. Gliomas are characterized by high invasiveness, high recurrence, poor clinical prognosis, and are associated

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Niansheng Lai, Hao Zhu, Yijun Chen, Shuai Zhang, Xudong Zhao, Yuchang Lin

with a disproportionately high morbidity and mortality [40,46]. According to the World Health Organization (WHO) classification scheme established in 2007 [18], astrocytic tumours are further sub-divided into grades: pilocytic astrocytoma (WHO grade I), diffuse astrocytoma (WHO grade II), anaplastic astrocytoma (WHO grade III), and glioblastoma (WHO grade IV). Oligodendroglial tumours are divided into oligodendroglioma and anaplastic oligodendroglioma.

MicroRNAs (miRNAs) are small (17-24 nucleotides) non-coding single-stranded RNAs, which regulate the expression of multiple target genes either by degrading specific mRNA or inhibiting translation [20]. To date, more than 2000 human miRNAs have been identified (http://www.mirbase.org/). They are involved in the regulation of multiple cellular and developmental processes, including cell proliferation, cell differentiation, cell cycle regulation, and epithelial-mesenchymal transition. miRNAs also play a role in the molecular pathology of cancer, including tumorigenesis, differentiation, proliferation, cell cycle regulation, angiogenesis, adhesion, migration, invasion and apoptosis of tumour cells [11]. miRNAs are often regarded as highly stable molecules but can undergo accelerated decay under certain environmental conditions allowing for maintenance of homeostasis through both biosynthetic and decay processes. Mitotically active cells, cells exposed to growth factors, and increases in neuronal activity are physiologic triggers known to induce a rapid decay of associated miRNA molecules. For example, the half-life of miR-29b is 4 hours in cycling cells but > 12 hours in mitotically arrested cells. Rapid degradation only affects certain subsets of miRNAs but appears to be a prevalent characteristic of miRNAs involved in neuronal activity [30]. Five different miRNAs tested in primary human neuron cultures were observed to all have half-lives less than 3.5 hours [3,30,32]. In multiple studies, miRNA expression levels have been analyzed as potential biomarkers for specific diagnosis and disease progression [17,36].

The miRNA, miR-210, is consistently identified in hypoxic events in both normal and transformed cells [5]. It was originally identified in cardiac cells undergoing a cardiac infarction. MiR-210 is up-regulated under hypoxia, which has been observed to increase angiogenesis and inhibit apoptosis [5]. MiR-210 has also been observed to be up-regulated in tumours. Hypoxia is a predominant feature of the tumour microenvironment of gliomas with more aggressive tumours leading to an increase in hypoxia. It has been demonstrated that miR-210 plays an important role in regulation of cell growth, angiogenesis, invasion and apoptosis in different human tumour models [5,11]. MiR-210 expression is frequently up-regulated in a variety of solid tumours, including breast cancer [12], non-small cell lung cancer [29], head and neck cancer [8], pancreatic cancer [4], oral tumours [31], hepatocellular cancer (HCC) [42], adrenocortical carcinoma (ACC) [23], glioblastoma [26], colon cancer [24], ovarian cancer [9], malignant melanoma [22] and renal cell cancer [27]. The expression of miR-210 is found to be down-regulated in human esophageal squamous cell carcinoma (ESCC) tissues and derived cell lines [34]. Because of the consistency of the presence of miR-210 in hypoxic environments, it is often utilized as a biomarker for glioma detection [16,21,41]. The aim of this study was to determine if there is a correlation between the grade of astrocytoma and miR-210 expression and if the origin of the glioma affects miR-210 expression.

Material and methods

Clinical samples

This study complied with the requirements of the Research Ethics Committee of Nanjing Medical University, P.R. China.

Eighty-one glioma samples were obtained from February 2010 to January 2013 in the Department of Neurosurgery of Wuxi Second People's Hospital. The diagnosis of primary gliomas was re-reviewed histologically by the experimental neuropathologist. Each sample was classified and scored based on the WHO standard for tumour classification [18] by two neuropathologists to ensure accuracy, with differences resolved by careful discussion. Fifty-eight of the 81 gliomas were classified as astrocytic tumours [10 diffuse astrocytomas (WHO grade II), 17 anaplastic astrocytomas (WHO grade III), and 31 glioblastomas (WHO grade IV)]. Twenty-three of 81 gliomas were classified as oligodendroglial tumours.

Glioma types were further confirmed through hematoxylin and eosin staining-based microscopic investigations after the surgery. Tissue samples were quickly resected, immediately snap-frozen in liquid nitrogen and stored at –80°C until RNA extraction. The study group of patients consisted of 37 women and 44 men, and the median age was 47 (15-77

years). None of the patients had received chemotherapy or radiotherapy prior to surgery. Ten snap-frozen samples of normal brain tissue were obtained from internal decompression of patients who underwent neurosurgery due to cerebral injury and cerebral haemorrhage. Informed consent was obtained before the surgery.

Isolation of RNA

Total RNA was extracted from frozen tissue with Trizol reagent (Invitrogen, Carlsbad, CA, USA), according to manufacturer's protocol. The RNA quantity and quality were measured by NanoDrop 2000 spectrophotometer (NanoDrop Technologies, Houston, TX, USA). Only samples with an OD A260/A280 ratio between 1.8 and 2.0 were utilized for further analysis by RT-PCR.

cDNA synthesis

The miR-210 and U6B (as an internal control)-specific cDNA was synthesized from 3-4 µg of total RNA with a mixture of miR-210-RT primers/U6B-RT primers (Genepharma, Shanghai, China) and M-MLV reverse transcriptase.

Real-time quantitative reverse transcription-PCR

Analysis of gene expression was performed by real time quantitative PCR (RT-PCR). RT-PCR was performed using the miR-210 primer set with the RT-PCR Master Mix and analyzed with the DNA Engine Opticon 2 Real-Time Cycler (MJ Research, Inc., Waltham, MA, USA) according to the manufacturer's instructions. Each reaction included 0.8 µl of miR-210 primer set (5 µM) (Genepharma), 20 µl of RT-PCR master mix (Genepharma), 0.2 µl of Taq DNA polymerase (5 U/µl) (Genepharma) and 4 µl of RT products. The quantitative analysis of the change in expression levels was calculated in triplicate using the comparative cycle threshold (CT) method. The raw data of target miRNA were normalized to U6B which served as an internal control.

Statistical analysis

All data were analyzed using IBM SPSS Statistics 13.0 for Windows (SPSS Inc., Chicago, IL, USA). The independent sample t-test was utilized to determine the statistical differences among the groups between glioma tissues and normal brain tissues. To determine the correlation between expression levels of miR-210 and histologic grade, the Spearman rank correlation test was applied. Differences were considered statistically significant when $p < 0.05$.

Results

MiR-210 expression in astrocytic tumours

The expression of miR-210 was detected in 58 astrocytic tumours and 10 normal brain tissues normalized to U6B by quantitative RT-PCR. As shown in Fig. 1, astrocytic tumours demonstrated a significant increase in miR-210 transcript levels compared with the mean expression levels observed in normal brain tissues (mean ± SD 6.91 ± 1.3 vs. 4.84 ± 0.35; $p < 0.001$). Expression levels of miR-210 in diffuse astrocytomas (WHO grade II; mean ± SD 5.69 ± 0.67; $p = 0.004$), anaplastic astrocytomas (WHO grade III; mean ± SD 6.46 ± 0.90; $p < 0.001$), and glioblastomas (WHO grade IV; mean ± SD 7.62 ± 1.12; $p < 0.001$) were significantly higher than those found in normal brain tissues.
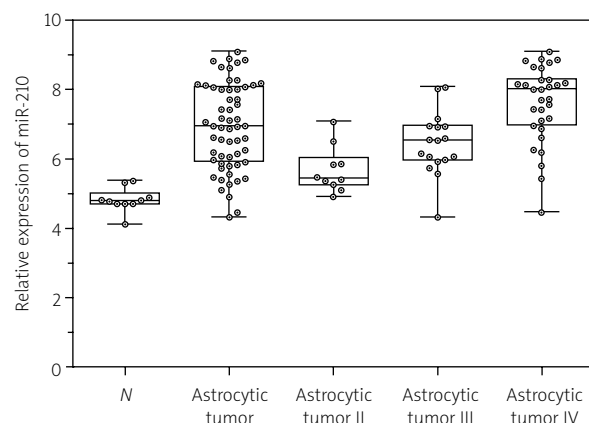
Fig. 1. MiR-210 expression in 58 astrocytic tumours and 10 normal brain tissues detected by real-time quantitative RT-PCR. Astrocytic tumours demonstrated a significant increase in miR-210 transcript levels compared with the mean expression levels observed in normal brain tissues (mean ± SD 6.91 ± 1.3 vs. 4.84 ± 0.35; $p < 0.001$). Expression levels of miR-210 in diffuse astrocytomas (WHO grade II; mean ± SD 5.69 ± 0.67; $p = 0.004$), anaplastic astrocytomas (WHO grade III; mean ± SD 6.46 ± 0.90; $p < 0.001$), and glioblastomas (WHO grade IV; mean ± SD 7.62 ± 1.12; $p < 0.001$) were significantly higher than those found in normal brains tissues.

Niansheng Lai, Hao Zhu, Yijun Chen, Shuai Zhang, Xudong Zhao, Yuchang Lin

Increase in miR-210 expression is directly correlated with the histopathological grade of the astrocytic tumour

Once it was determined that astrocytic tumours expressed a significantly higher level of miR-210 than normal brain tissue, we wanted to determine if there was a statistically significant correlation between the histopathological grade of the tumour and level of miR-210 transcript. There was a significant increase in miR-210 expression in glioblastomas compared with anaplastic astrocytomas ($p < 0.001$), glioblastomas compared with diffuse astrocytomas ($p < 0.001$), and anaplastic astrocytomas compared with diffuse astrocytomas ($p < 0.05$) (Fig. 1). According to the Spearman rank correlation test, increasing levels of miR-210 expression could be directly correlated with a higher pathological grade. In other words, there was a positive correlation between miR-210 expression and pathological grade ($r = 0.646$, $p < 0.001$). No statistically significant association between miR-210 expression and age at diagnosis, size of tumours, gender of patients, or KPS score could be identified ($p > 0.05$, Table I).

**Table I.** Association between miR-210 expression and different clinicopathological features of astrocytic tumours

Clinicopathological parameters	No. of cases	miR-210 expression	p value
Sex			
Male	32	6.86 ± 1.27	0.55
Female	26	7.06 ± 1.23	
Age			
< 55	28	6.93 ± 1.21	0.912
≥ 55	30	6.97 ± 1.30	
WHO grade			
II	10	5.69 ± 0.67	< 0.001
III	17	6.46 ± 0.90	
IV	31	7.62 ± 1.12	
Tumour size			
≥ 5 cm	23	6.84 ± 1.21	0.58
< 5 cm	35	7.02 ± 1.28	
KPS			
< 90	25	6.90 ± 1.15	0.80
≥ 90	33	6.99 ± 1.33	

MiR-210 expression in oligodendroglial tumours

To determine if miR-210 expression was increased in gliomas of different origins, the expression level of miR-210 was analyzed in 23 oligodendroglial tumours and compared to levels in 10 normal brain tissues normalized to U6B. The results demonstrated that the expression of miR-210 was significantly reduced in oligodendroglial tumours, compared with the mean expression levels observed in normal brain tissues (Fig. 2; mean ± SD 3.78 ± 0.76; $p < 0.01$).

Discussion

Altered expression levels of miRNAs have been observed in gliomas, appearing to have both an oncogenic role as well as a tumour suppressor role. More than 1000 studies in cancer and more than 100 studies in gliomas on associated miRNAs have been published to date [44]. Most of these studies examine the expression levels, targets and functional effects of selected miRNAs in glioma cells and tissues. Some miRNAs that are highly expressed in glioma tumours have been implicated in up-regulating cell processes such as cell growth and proliferation, migration, invasion, angiogenesis and cell transformation and down-regulating apoptosis. Examples of these miRNA include: miR-21, miR-221/222, miR-
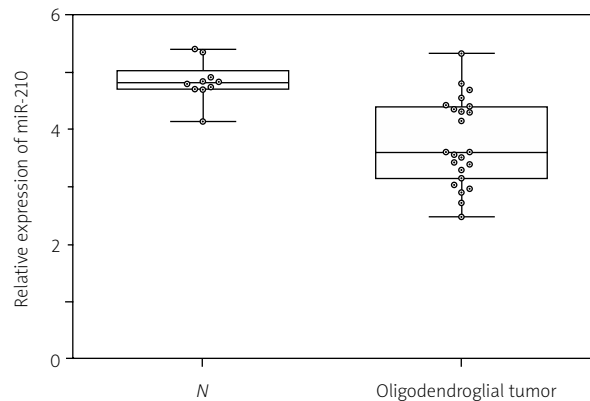
Fig. 2. MiR-210 expression in 23 oligodendroglial tumours and 10 normal brain tissues detected by real-time quantitative RT-PCR. Oligodendroglial tumours demonstrated a significant decrease in miR-210 transcript levels compared with the mean expression levels observed in normal brain tissues (mean ± SD 3.78 ± 0.76 vs. 4.84 ± 0.35; $p < 0.01$).

210, miR-9, miR-10(a,b), miR-15(a,b) and miR-26a. MiRNA expression can also be reduced in gliomas, acting as tumour repressors, decreasing cell growth and proliferation, migration and invasion and increasing apoptosis, such as seen with miR-181(a,b,c), miR-128, miR-451, miR-7, miR-34a, miR-124, miR-218 and miR-326 [1,14,27,33,38,44]. There have also been studies profiling miRNA levels in gliomas in order to compare and determine potential differences between tumour grades. Currently there is an assay to analyze a 23-miRNA signature that differentiates between glioblastomas and anaplastic astrocytomas with 95% accuracy [44]. Changes in miRNA expression patterns can serve as informative biomarkers indicating the functional status of a normal brain as well as disease progression and prognosis of brain tumours [13,35].

By combining miRNA expression profiles with conventional radio/chemotherapy, treatment efficiency could be improved leading to a reduction in tumour recurrence after surgical resection [20]. Recently, a therapeutic scheme involving incorporation of artificial miRNAs in an oncolytic herpes simplex virus achieved successful silencing of specific targets in patients with melanoma and provides a new method for using miRNA as a potential therapeutic target [1]. In gliomas, down-regulation of miR-21 increases glioma apoptosis, represses cell growth, and induces cell cycle arrest. Anti-sense miR-21 was utilized in combination with 5-fluorouracil to target the human glioma cell line U251, significantly increasing apoptosis and decreasing cell migration [28].

MiR-210 is consistently induced under conditions of hypoxia [5], and plays an important role in many tumorigenic processes, such as cell proliferation [10], metabolism [39], cell migration and angiogenesis [6]. A number of targets of miR-210 have been reported, such as vacuole membrane protein 1 (VMP1), enzyme glycerol-3-phosphate dehydrogenase 1-like (GPD1L), iron-sulfur cluster scaffold homolog (ISCU), succinate dehydrogenase complex subunit D (SDHD) and MNT [15,19,25,42,45].

MiR-210, under hypoxic conditions, is frequently increased in HCC and is involved in hypoxia-induced HCC metastasis. VMP1, a putative metastasis suppressor in HCC, is identified as a direct and functional target of miR-210. Down-regulation of VMP1 by miR-210 mediates hypoxia-induced HCC migration and invasion [42].

A hypoxia-induced positive feedback loop promotes hypoxia-inducible factor 1α (HIF-1α) stability through miR-210 suppression of GPD1L [15]. Preventing degradation of HIF-1α leads to induction of a wide variety of genes including those involved in energy metabolism, angiogenesis, cell proliferation and survival [15]. In addition, HIF-1α stability leads to further up-regulation of miR-210 [15,19].

In renal tumours, miR-210 expression is increased under hypoxia by HIF-1/2. ISCU is repressed in the presence of miR-210 leading to a decrease in Krebs cycle enzyme activity and mitochondrial function, providing a mechanism for handling increases in free radical production and a switch to glycolysis affecting cell growth and survival under hypoxic conditions. A decrease in ISCU in cancer patients has been associated with a worse prognosis and is therefore important as a clinicopathological prognostic factor [7,19]. MiR-210's induction of mitochondrial dysfunction under hypoxia is further supported by down-regulation of SDHD in the presence of miR-210, which leads to an increase in HIF-1 stability in lung tumour cells, A549 [25].

MiR-210 constitutes a negative feedback of the HIF hypoxic response by reducing MNT and thereby increasing MYC transcription factor activity, which is known to enhance cell proliferation. An increase in miR-210 and therefore a sustained increase in MYC activity can be correlated with metastatic behaviour in tumours [45]. These examples demonstrate that miR-210 plays a key role in tumour progression and provides vital information about the tumour microenvironment. In addition to being a consistent biomarker, miR-210 could also be an excellent therapeutic target.

The expression of miR-210 has been shown to be correlated with a poor outcome in a variety of solid tumours, such as breast cancer [12], head and neck cancer [8], renal cancer [19], paediatric osteosarcoma [2] and acute ischemic stroke [43]. Meta-analysis has found that a high expression of miR-210 was an independent factor indicating a poor prognosis in 511 cases of breast cancer [12]. MiR-210 up-regulation showed a strong correlation with tumour aggressiveness in paediatric osteosarcoma, with a significant reduction in both overall survival and progression-free survival [2].

Results of the current study indicate that miR-210 will also be an appropriate biomarker and disease progression indicator for glioma tumours. We had

the opportunity to perform a large retrospective study to determine if there is a correlation between miR-210 expression and pathologic features and the clinical outcome of patients with different gliomas. By analyzing 81 clinical primary glioma tissues and 10 non-neoplastic brain tissues, we demonstrated that the expression of miR-210 is variable depending on the origin of the glioma. MiR-210 was found to be over-expressed in astrocytic tumour, whereas expression was down-regulated in the oligodendroglial tumours. We do not currently understand the biological relevance of the differential regulation between these two types of gliomas. Oligodendroglial tumours do possess different upstream regulatory genes and proteins and miR-210 may have simply evolved alternative regulatory targets. Additional research will be necessary to determine if miR-210 is a relevant biomarker for oligodendroglial tumours and what its potential role in tumour progression is. Moreover, the expression of miR-210 increased as the grade of astrocytic tumour increased.

In conclusion, miR-210 can be used as an auxiliary identification method for pathological identification of glioma origin, as well as a biomarker for malignant progression of astrocytic tumours. In future studies we hope to determine if miR-210 plays a regulatory role in tumour progression and evaluate its potential as a therapeutic target.

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