J Neurooncol (2009) 93:325–332
DOI 10.1007/s11060-009-9797-4

LABORATORY INVESTIGATION - HUMAN/ANIMAL TISSUE

miR-21 and 221 upregulation and miR-181b downregulation in human grade II–IV astrocytic tumors

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Received: 25 October 2008 / Accepted: 4 January 2009 / Published online: 22 January 2009

Abstract MicroRNAs (miRNAs) are small noncoding regulatory RNAs that reduce stability and/or translation of fully or partially sequence-complementary target mRNAs. Recent evidence indicates that miRNAs can function both as tumor suppressors and as oncogenes. It has been demonstrated that in glioblastoma multiforme miR-21 and 221 are upregulated whereas miR-128 and 181 are downregulated. Expression of miR-21, 221, 128a, 128b, 128c, 181a, 181b, 181c was studied using real-time quantitative reverse transcriptase polymerase chain reaction and northern blotting for human astrocytic tumors with different grade of malignancy. miR-21 and 221 were overexpressed in glioma samples, whereas miRNA 181b was downregulated compared with normal brain tissue. miRNA-21 was hyperexpressed in all tumor samples whereas higher levels of miRNA-221 were found in high-grade gliomas. This study is the first analysis of miRNAs in astrocytic tumor at different stages of malignancy. The different expression pattern observed in tumors at different stages of malignancy is probably dependent on the cell-specific repertoire of target genes of tumors sharing different molecular pathways activity and suggests miRNAs may have also a place in diagnosis and staging of brain tumors.

Keywords MicroRNA · miR-21 · miR-221 · miR-181 · Glioma · Brain tumor

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Background

MicroRNAs (miRNAs) are a recently discovered class of small, evolutionary conserved, RNA molecules that negatively regulate gene expression at the post-transcriptional level. The discovery of these small noncoding transcripts has broadened our understanding of the mechanisms that regulate gene expression, adding an entirely novel level of regulatory control.

miRNAs consist of 18–25 nucleotides and are a class of endogenous ribo-regulators that modulate gene expression via the RNA interference (RNAi) pathway. RNAi is a post-transcriptional silencing mechanism, present in most eukaryotic organisms, in which exposure to double-stranded RNA induces the sequence-specific degradation of homologous messenger RNAs (mRNA). miRNAs act by base-pairing with their target mRNAs through perfect or nearly perfect complementarity particularly at the 3′ untranslated regions (UTRs) of the target mRNAs [1–5] leading to their translational repression and/or direct cleavage [6].

To understand the mechanism of miRNA-mediated silencing, the basic step of their biogenesis must be summarized. miRNAs originate from long primary miRNAs (pri-miRNAs) that are transcribed in the nucleus by the RNA polymerase II complex. miRNAs are then processed

to 60–70-nucleotide precursor miRNA (pre-miRNA) intermediates by a complex of the RNase III enzyme DROSHA and a double-stranded RNA binding domain possessing the protein DGCR8 (DiGeorge syndrome critical region gene 8). These hairpin-shaped pre-miRNAs are transported to the cytoplasm by exportin-5 (Exp5, a member of the Ran transport receptor family). Once in the cytoplasm, they are cleaved by DICER to generate 20–22-nucleotide duplexes bearing two nucleotide single-stranded 3′ extensions. Finally, the miRNA–miRNA* duplex is unwound into mature miRNA and miRNA* by a helicase. Single-strand miRNA is incorporated into a ribonucleoprotein effector complex, known as the RNA-induced silencing complex (RISC), whereas miRNA* is degraded [7–9]. RISC identifies target messages based on complementarities between the "guide" miRNA and the mRNA, and results in either endonucleolytic cleavage of targeted mRNA or translational repression.

There are estimates, in part based on computational methods, that mammalian genomes encode up to 1,000 unique miRNAs [10], which are predicted to regulate expression of as much as 30% of genes [11]. Although more than 530 miRNAs have been identified in humans, much remains to be understood about their precise cellular function and role in the development of diseases. Because miRNAs are implicated in the control of many fundamental cellular and biological processes, for example developmental timing [12], stem cell division [13, 14], and apoptosis [15], changes in their expression may play a role in the development of diseases [16, 17] and cancer [6, 18]. Recent evidence indicate that miRNAs can function both as tumor suppressors and oncogenes [19, 20]. Indeed, altered miRNA expression has been reported in leukemia, lung cancer, colon cancer, and breast cancer [21–23], and in many other malignancies.

A role of miRNA has also been suggested in glioblastoma (GBM), the most common tumor of the brain and one of the most aggressive tumor in humans [24]. Ciafre et al. studied the global expression of 245 microRNAs in GBM using a microarray technique [25]. This approach enabled the identification of miRNAs whose expression is significantly altered in tumors compared with peripheral brain areas from the same patient, including miR-221, strongly up-regulated in glioblastoma, and a set of brain-enriched miRNAs, miR-128, miR-181a, miR-181b, and miR-181c, which were down-regulated in glioblastoma. Chan et al. [26] used oligonucleotide arrays specific for 180 human and mouse miRNAs and found marked elevation of miR-21 levels in human primary GBM tissues and cell lines.

Nevertheless, nothing is known about the expression of miRNAs in astrocytic tumors at earlier stages of malignancy. In this study we analyzed the expression of the abovementioned set of miRNAs in human astrocytic tumors at different stages of malignancy.

Material and methods

Patient population

This study included tumor samples histologically verified as grades II–IV astrocytomas obtained from adult patients who had undergone craniotomy for microsurgical tumor removal. All tumors were located in the supratentorial compartment. Only patients who had undergone extensive gross-total resection of the neoplasm (95% of the tumor volume) were eligible to participate in the study. We excluded oligodendroglial tumors, recurrent tumors, and patients who had undergone neoadjuvant therapy (radiotherapy and/or chemotherapy) before surgery. Furthermore, four samples of normal brain tissue were used as controls.

The study included 16 men and 12 women whose mean age was 55.4 ± 16.5 years at surgery (range 27–76 years). The Karnofsky performance status (KPS) scores in these patients was 91.1 ± 12.6 (range 60–100). According to the revised World Health Organization (WHO) classification [27], tumors were diagnosed as low-grade astrocytoma (LGA; eight patients), anaplastic astrocytoma (AA; ten patients), and glioblastoma multiforme (GBM; ten patients). Patients with LGAs had a mean age of 37.1 ± 6 years, a KPS score of 100, and a survival period longer than 208 weeks. Patients with AAs had a mean age of 62.1 ± 15.4 years, a mean KPS score of 86 ± 16.5, and a mean survival period of 109.3 ± 17.9 weeks (median 112 weeks). Patients with GBMs had a mean age of 51.4 ± 12.2 years, a mean KPS score of 89 ± 9.9, and a mean survival period of 51.4 ± 9.9 weeks (median 54 weeks). The total duration of follow up was 208 weeks postsurgery.

Tissue samples

All tumor tissue samples were obtained from the neoplastic tissue within 15 min of surgical removal. Neoplastic tissue samples were taken from viable areas of tumor, while trying to avoid areas of gross necrosis. Three anatomically separate areas of the tumor were sampled from each resected specimen. Non-neoplastic brain tissue samples were derived from the temporal lobes of patients surgically treated for temporal lobe epilepsy and included histologically verified normal cortex and white matter. Tissue samples for analysis were placed in cryovials and immediately flash frozen in liquid nitrogen in the operating room and stored at −80°C. Tissue adjacent to the frozen samples and additional tissue obtained from the resection specimens

were formalin-fixed, paraffin-embedded, cut, and stained with H & E and used for histological typing and grading on the basis of the WHO criteria.

RNA extraction

Enriched microRNAs were extracted using the mirVana miRNA Isolation kit (Ambion, Italy), and total RNA was also extracted using a total nucleic acid Isolation kit (Ambion) and following the manufacturer's procedure. The concentrations of samples were measured spectrophotometrically and the quality was checked on agarose gel.

Reverse transcriptase reactions

Reverse transcriptase reaction contained RNA samples including purified miRNA, 50 nM stem-loop RT primer of each miRNA (RNU6, 21, 128a, 128b, 128c, 181a, 181b, 181c, and 221; purchased from Applera, Italy), 0.25 mM each of dNTPs, 3.33 U/μl MultiScribe reverse transcriptase (P/N: 4319983; Applied Biosystems, Milan, Italy) and 0.25 U/μl RNase inhibitor (P/N: N8080119; Applied Biosystems, Milan, Italy). The 7.5 μl reactions were incubated in an thermocycler for 30 min at 16°C, 30 min at 42°C, 5 min at 85°C, and then held at 4°C. All reverse transcriptase reactions, including no-template controls and RT minus controls, were run in duplicate.

Real-time PCR

Real-time PCR was performed using a standard TaqMan PCR kit procedure on an Applied Biosystems 7300 real-time PCR system (Applied Biosystems). The 10 μl PCR included 0.67 μl RT product, 1× TaqMan Universal PCR Master Mix (P/N: 4324018, Applied Biosystems), and 0.2 μM TaqMan probe. The reactions were incubated in a 96-well plate at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. All reactions were run in triplicate. The threshold cycle (C_T) is defined as the fractional cycle number at which the fluorescence passes the fixed threshold. TaqMan C_T values were converted into absolute copy numbers using a standard curve from miRNA U6.

Northern blot analysis

RNA samples (10 μg each) were electrophoresed on 15% acrylamide and 7 mol/l urea Criterion precast gels (Bio-Rad) and transferred on to Hybond N+ membrane (Amersham Biosciences). Membranes were hybridized with oligonucleotide probes corresponding to the complementary sequences of the below indicated mature miRNAs. The oligo probes were designed on the basis of individual miRNA sequence information deposited in miRBase (http://microrna.sanger.ac.uk):

miR-21, 5′-ATCGAATAGTCTGACTACAACT-3′,
miR-128-a, 5′-AGTGTCACTTGGCCAGAGAAAA-3′,
miR-128-b, 5′-AGTGTCACTTGGCCAGAGAAAG-3′,
miR-128-c, 5′-TTGTAAGTTGGACAGCCACTCA-3′,
miR-181-a, 5′-TTGTAAGTTGCGACAGCCACTCA-3′,
mir-181-b, 5′-TTGTAAGTAACGACAGCCACCC-3′,
mir-181-c, 5′-AACAUUCAACCUGUCGGUGAGU-3′ and
miR-221, 5′-GAAACCCAGCAGACAATGTAGCT-3′.

Probes were 5′-end labeled using the polynucleotide kinase in the presence of [γ-^{32}P]ATP. Hybridization was performed at 37°C in 7% SDS/0.2 mol/l Na_2PO_4 (pH 7.0) for 16 h. Membranes were washed at 42°C, twice with 2× standard saline phosphate (0.18 mol/l NaCl/10 mmol/l phosphate, pH 7.4), 1 mmol/l EDTA (saline-sodium phosphate-EDTA; SSPE), and 0.1% SDS, and twice with 0.5× SSPE/0.1% SDS. Northern blots were rehybridized after stripping the oligonucleotides used as probes in boiling 0.1% SDS for 10 min. As a control for normalization of RNA expression levels, we hybridized blots with an oligonucleotide probe complementary to the *U6 RNA* (5′-GCAGGGGCCATGCTAATCTTCTCTGTATCG-3′). After blotting, results were visualized on Kodak X-ray film (Kodak, Milan, Italy) by autoradiography.

Statistical data analysis

Statistical analysis was accomplished using the unpaired Student *t*-test with Welch correction to compare the expression levels of miR-21, 221, 128a, 128b, 128c, 181a, 181b, and 181c as quantified on real time RT-PCR in LGAs, AAs, and GBMs. Data analysis was performed with INSTAT, version 3.0, and PRISM, version 4.0 (Graphpad, San Diego, CA, USA).

Results

To identify deregulated miRNAs we used quantitative real time-PCR (Taqman) to measure expression of eight mature miRNA sequences in human non-neoplastic brain tissues (NBT), LGAs (WHO grade II); AAs (grade III), and GBMs (grade IV). The comparative Ct (ΔΔCt) method was used to determine the change in expression of each miRNA in tumor samples relative to that in normal brain tissue. Briefly, the ΔCt of each miRNA was determined relative to U6 endogenous control RNA, which was robustly and invariantly expressed across all samples, and the average ΔCt of the four NBT samples was used as the calibrator for the tumor samples.

miR-21 was up-regulated seven to elevenfold in tumor samples. In detail miRNA-21 changes over NBT were: 8.76 ± 1.25-fold in LGAs, 9.39 ± 1.46-fold in AAs, and 9.18 ± 2.54-fold in GBMs (Fig. 1a).

miR-221 was consistently upregulated in our sample set. miRNA-221 was up-regulated 5 to 17-fold in tumor samples. In detail, miRNA-21 changes over NBT were: 7.69 ± 1.43-fold in LGAs, 9.1 ± 2.4-fold in AAs, and 12 ± 2.5-fold in GBMs (Fig. 1b).

Expression of miR-181b decreased by a factor of 6 to 17. In detail, miRNA-181b changes over NBT were: a factor of 0.1 ± 0.03 in LGAs, a factor of 0.13 ± 0.03 in AAs, and a factor of 0.09 ± 0.03 in GBMs (Fig. 1c).

Analysis of miRNA-128a, 128b, 128c, 181a, 181c showed expression levels were within threefold those of NBT and were considered not significantly changed.

We next performed statistical analyses of our miRNA expression data to identify differences in expression among tumor sample with different grades of malignancy. There were no differences in expression levels of miRNA-21 and miRNA-181b among LGAs, AAs, and GBMs. miRNA-221 was significantly more up-regulated in GBMs compared with LGAs ($P < 0.01$) and AAs ($P < 0.01$) (Fig. 1d).

miRNAs were further assayed by Northern blot for validation purposes (Fig. 2). miRNAs expression was normalized with the corresponding U6 RNA in each sample. Northern blot analysis confirmed the results obtained by quantitative RT-PCR displaying similar expression pattern. In particular, northern blot analysis revealed that miR-21 was up-regulated in all glioma samples. miR-221 was significantly up-regulated in GBMs compared with LGAs.

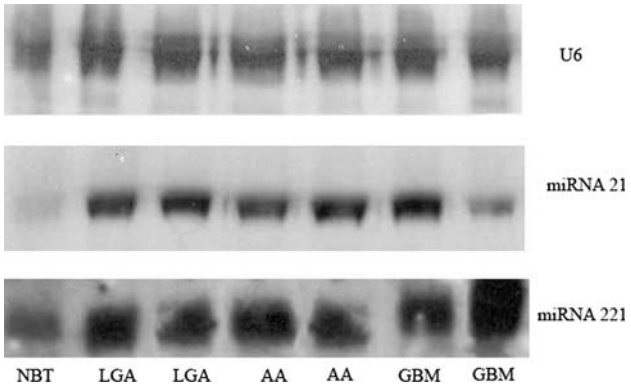

Fig. 2 Representative northern blot autoradiographs for internal control RNA U6 (*upper*), miRNA-21 (*middle*), and miRNA-221 (*lower*) expression studied in normal brain tissue and human gliomas. *NBT*, normal brain tissue; *LGA*, low-grade glioma; *AA*, anaplastic astrocytoma; *GBM*, glioblastoma multiforme

Discussion

Our results showed that miRNA 21 and 221 are up-regulated in human astrocytic tumors, whereas miRNA 181b is

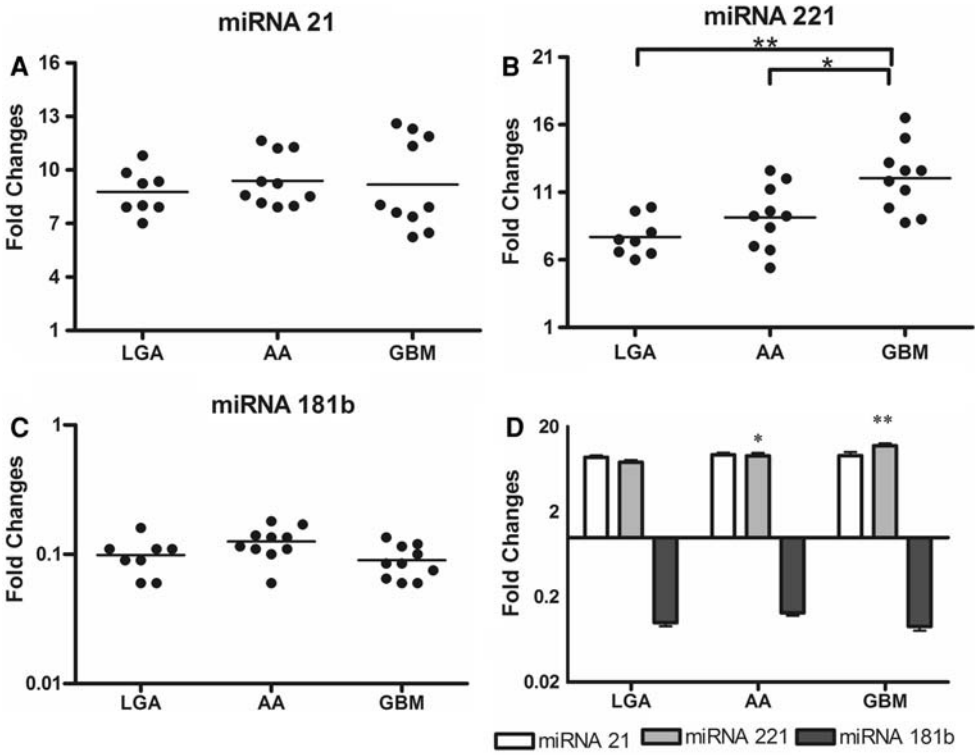

Fig. 1 Real-time quantitative RT-PCR expression was performed on cDNA from patient samples for miR-21, miR-221, and miR-181b. The expression levels of each gene were calculated as multiples of their expression in normal brain tissue and depicted for each individual sample per disease type. **a** miR-21 was up-regulated 7 to 11-fold in tumor samples. **b** miRNA-221 was up-regulated 5 to 17-fold. **c** miR-181b expression was down-regulated by a factor of 6 to 17 in tumors compared with NBT. **d** Global representation of deregulated miRNA in the three different histotypes is also displayed. *$P < 0.05$; **$P < 0.01$. *LGA*, low-grade glioma; *AA*, anaplastic astrocytoma; *GBM*, glioblastoma multiforme

down-regulated. miRNA-21 was homogeneously overexpressed in low and high-grade tumors whereas miRNA-221 overexpression was more evident in high-grade tumors.

The discovery of miRNAs dates back to 1993 when Lee et al. described a small RNA, lineage-deficient-4 (lin-4), with antisense complementarity to lin-14 involved in the regulation of developmental timing in *Caenorhabditis elegans* [28]. At that time most investigators considered this small RNA as an oddity in worm genetics, but hundreds of these miRNAs were soon discovered in *C. elegans* and other animals by different laboratories [29–32]. There is currently evidence of expression of 340, 303, and 205 distinct mature miRNAs in human, mouse, and rat encoded by 395, 363, and 231 different miRNA genes, respectively [33].

MicroRNAs may play a role in the tumorigenesis and progression of cancer. Calin et al. first established a connection between microRNAs and cancer by showing that miR-15 and miR-16 are located on chromosome 13q14, a region deleted in more than half of B-cell chronic lymphocytic leukemia (CLL) [34]. Cimmino et al. then demonstrated that expression of miR-15a and miR-16-1 was inversely correlated with Bcl-2 expression in CLL and that both miRNAs negatively regulated Bcl-2 at a post-transcriptional level. Furthermore, Bcl-2 repression by these miRNAs induced apoptosis in a leukemic cell line model [15].

Our results agree with previous observations that miRNA-21 is overexpressed in some cancer histotypes, including gliomas. MicroRNA-21 is overexpressed in breast cancer [35, 36], and pancreatic cancer [37]. The first study on the possible involvement of miRNA in glioma revealed hyperexpression of miRNA-21 [26]. It showed markedly elevated miR-21 levels in human glioblastoma tumor tissues, in early-passage glioblastoma cultures, and in six established glioblastoma cell lines (A172, U87, U373, LN229, LN428, and LN308) compared with non-neoplastic fetal and adult brain tissues and compared with cultured nonneoplastic glial cells. Furthermore, the authors demonstrated that knockdown of miR-21 in cultured glioblastoma cells triggered the activation of caspases leading to increased apoptosis [26]. We observed that miRNA-21 is significantly upregulated in LGAs. These tumors are characterized by a low rate of proliferation and prolonged life-span, which suggests that defective apoptosis pathways may play a prominent role in the pathogenesis of such tumors.

Even though an antiapoptotic role is likely for miR-21 in gliomas, other mechanisms are possible, because in Hela cells its down-regulation determines, instead, an increase in cell proliferation without affecting apoptosis [38]. Meng et al. demonstrated that knock-down of miR-21 may render cholangiocarcinoma cells sensitive to gemcitabine whereas transfection of non-malignant cholangiocytes with the precursor of miR-21 renders them more resistant to this drug [39]. This may be the result of downregulation of *PTEN*, the phosphatase that antagonizes the growth-promoting activity of the PI-3 kinase-Akt signaling pathway. It is worthy of note that *PTEN* was originally cloned as a tumor suppressor for brain tumors with point mutations occurring in 25% of cases [40].

Further work by Frankel et al. on breast cancer cells [41] and by Asangani et al. [42] on colon cancer cells found that miR-21 overexpression leads to PDCD4 (programmed cell death 4) reduction, by direct interaction with its 3′ UTR, and that anti-miR-21 treatment is followed by an increase in endogenous PDCD4 protein levels. PDCD4 is a tumor suppressor known to be up-regulated during apoptosis [43] and reduced in different tumors [44], and evidence demonstrates that miR-21 overexpression effects are at least in part due to PDCD4 downregulation. Although this target has not been proven in brain tumors, it is possible that it may be relevant in these tumor types also, as a recent study demonstrated that most of the glioma samples analyzed lacked PDCD4 protein expression whereas adjacent normal glial tissues expressed high levels of PDCD4 [44]. An additional molecular mechanism, proving the oncogenic properties of miR-21 in an in-vivo xenograft breast cancer model, consists in targeting of the tumor suppressor protein tropomyosin 1 (TPM1) at the translational level. Tropomyosin 1 is able per se to reduce tumor cell proliferation and anchorage independence, therefore explaining the effects on tumor growth exerted by miR-21 [45].

A recent publication explored the expression of miRNAs in GBM, using microarray analysis to determine the global expression of miRNAs in tumors relative to normal brain [25]. Ciafre et al. confirmed the upregulation of miR-21, but also identified an additional set of upregulated miRNAs. Notably, amongst these was miR-221, which was up-regulated in a subset of glioblastoma samples and in all glioblastoma cell lines examined. Our results confirm the hyperexpression of miR-221 in glioma.

The role of miR-221 in tumor development has been also analyzed. Gillies and Lorimer defined a specific function of miRNA 221 and 222 in glioblastoma, showing that they repress expression of the cell cycle regulatory protein $p27^{Kip1}$ [46]. The $p27^{Kip1}$ gene is a member of the Cip/Kip family of cyclin-dependent kinase (CDK) inhibitors that function to negatively control cell cycle progression. The protein binds to CDK2 and cyclin E complexes to prevent cell cycle progression from G1 to S phase. $p27^{Kip1}$ also acts as a tumor suppressor and its expression is often disrupted in human cancers. Analysis of the 3′ UTR of $p27^{Kip1}$ suggests that repression of this protein is a consequence of direct binding of miRNA 221 and 222 to sites in the 3′ UTR.

This mechanism was suggested contemporaneously and independently by le Sage et al. who demonstrated, using miRNA inhibitors, that some glioblastoma cell lines require high activity of miR-221 and 222 to maintain low $p27^{Kip1}$ levels and continuous proliferation, and that high levels of miR-221 and 222 seem to correlate with low levels of $p27^{Kip1}$ protein in GBM [47].

Gonzalez et al. have previously shown that inhibition of cdk4 activity enhances translation of $p27^{Kip1}$, providing a link between these two cell-cycle regulators [48]. This effect was shown to be mediated by the 3′ UTR of $p27^{Kip1}$. The glioblastoma cells used in that study were mutated at the INK4A/ARF locus and did not express the cdk4 inhibitor $p16^{Ink4a}$ [49]. This suggested the possibility that loss of cdk4 inhibition through INK4a/ARF mutations causes an increase in miR-221 levels, and a consequent decrease in $p27^{Kip1}$ levels.

According to those observations, the up-regulation of miRNA-221 in high-grade gliomas, recorded in our study, may suggest a specific role in the defective cell cycle control and high proliferation rate of those tumors compared with less malignant tumors. $p27^{Kip1}$ is a well-known tumor suppressor down-regulated in many human tumors, and its expression levels in primary cancers are highly correlated with reduced patient survival [50]. Similarly, miR-221 has also been associated with poor prognostic miRNA signatures in thyroid papillary carcinomas [51, 52], pancreatic adenocarcinoma, and GBM [25, 37], indeed reinforcing interconnection between miR-221 and $p27^{Kip1}$ in the pathogenesis of GBM.

Ciafre et al. in their microarray analysis of the global expression of miRNAs in glioblastoma found that miRNA-181a and miRNA-181b were downregulated in glioma specimens compared with non-tumoral adjacent tissue and in glioma cell lines compared with normal brain tissue [25]. There is limited but substantial evidence regarding the role of miRNA-181 in cancer. Cheng et al. showed that inhibition of miRNA-181a increases cell growth in the lung carcinoma cell line, A549 [53]. Pekarsky et al. found miRNA-181b was down-regulated in aggressive B-cell chronic lymphocytic leukemia with 11q deletion compared with the indolent form of chronic lymphocytic leukemia [54]. All this evidence seem to suggest that downregulation of miRNA-181 may play a role in cancer development. Very recently, Shi et al. reported the downregulation of miR-181a and 181b in both human gliomas and glioma cell lines, confirming the data of Ciafre et al. [55]. Those authors showed that transfection of those miRNAs triggered growth inhibition, apoptosis, and inhibited invasion. Furthermore, miR-181a expression turned out to be inversely correlated with tumor grading whereas miR-181b was uniformly downregulated in gliomas with different grades of malignancy. Our results partially confirm those observations and show significant and uniform downregulation of the miRNA-181b in human gliomas, with miRNA-181a showing a tendency toward downregulation which did not reach statistical significance. This difference was possibly because of the threshold selected to define the overexpression of miRNA, which was higher our study. Nonetheless, our results are based on a relatively small number of tumor samples, this limits their applicability and requires further studies.

Conclusions

In this study, we carried out an analysis of a set of miRNAs in astrocytic tumors at different stages of malignancy. Our results suggest that miRNA-21 is upregulated even in low-grade tumors whereas miRNA-221 upregulation may be a molecular mechanism characteristic of a higher grade of malignancy. The different expression pattern observed in tumors at different stages of malignancy is likely to be dependent on the cell-specific repertoire of target genes of tumors sharing different molecular pathways activity and suggests miRNAs may also have a place in diagnosis and staging of brain tumors. The demand for reliable markers suitable for prediction of prognosis is high, especially for cancer types such as gliomas. Further studies to fully elucidate the expression patterns of miRNAs in astrocytic tumors with different grades of malignancy are warranted.

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