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RESEARCH

Analysis of hsa-miR-30a-5p Expression in Human Gliomas

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Abstract Our previous study demonstrated that miR-30a-5p was upregulated in six malignant glioma cell lines by microRNA(miRNA) array. For further verification of this finding, the expression of miR-30a-5p in 7 more malignant glioma cell lines, 43 freshly resected glioma samples and 75 archival paraffin embedded glioma specimens with different grade of malignancy were examined by qRT-PCR and in situ hybridization(ISH). Here, we present the first evidence that miR-30a-5p is overexpressed in glioma cell lines and glioma samples as compared to the normal brain tissues (NBTs), and its expression level is positively correlated with tumor grade of malignancy. It is concluded that miR-30a-5p may have the potential as a diagnostic or prognostic marker of gliomas and as the target of miRNA-based glioma therapy in further studies.

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Introduction

Glioblastoma (GBM) is the most common and aggressive primary brain tumor in adults. Despite the improvement of current available treatment modalities, including surgical resection, radiotherapy and chemotherapy, the prognosis of patients with GBM remains grim. Most of the patients die of tumor recurrence with a median survival of 9–12 months [1–3]. Thus, it is imperative to develop novel strategies for the treatment of GBM.

MiRNAs are a class of short noncoding regulatory RNAs which act as posttranscriptional regulators of gene expression [4–6]. By binding to the 3'-untranslated region of target mRNAs, miRNAs could trigger translational downregulation and/or increase degradation of mRNA from target genes [7]. MiRNAs are involved in diverse biological processes, such as development, differentiation, cell proliferation and apoptosis. However, it remains uncertain whether altered miRNA expression is a cause or consequence of pathological processes. Although bioinformatic approaches can predict thousands of genes that are potentially targeted and regulated by miRNAs based on sequence complementarity, However, only a small fraction of miRNA targeted genes has been functionally validated.

MiRNAs have been suggested to contribute to the development of cancer [8]. Calin et al showed that half of the known miRNAs are located in cancer-associated genomic regions/fragile sites, thus potentiating their role in cancer [9], and they firstly established a connection between miRNAs 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) [10]. Hayashita et al found for the first time that the miR-17-92 cluster, which

comprises seven miRNAs and resides in intron 3 of the C13orf25 gene at 13q31.3, is markedly overexpressed in lung cancers, especially with small-cell lung cancer histology [11]. In recent years, deregulation of miRNAs has been observed in various types of human malignancy, including lymphoma, colorectal cancer, lung cancer, breast cancer, papillary thyroid carcinoma, hepatocellular carcinoma and glioblastoma [12–18].

We previously carried out a microRNA array of six glioma cell lines in which some microRNAs significantly upregulated as compared to NBTs, such as miR-21, miR-221/222, and miR-30a-5p, etc(data not shown), among which the involvement of miR-30a-5p in gliomas has not been reported before. Therefore, in the present study we aim to further analyze the expression of miR-30a-5p in human glioma cell lines and glioma samples with different grade of malignancy, and explore possible role of miR-30a-5p in gliomas.

Materials and Methods

Cell Culture

The U251, LN229, SNB19, LN308 and U87 human glioblastoma cell lines were purchased from the Institute of Biochemistry and Cell Biology, Chinese Academy of Science. Human glioblastoma cell lines, TJ899 and TJ905, were established and characterized in Laboratory of Neuro-oncology, Tianjin Neurological Institute [19]. Cells were maintained in Dulbecco's Modified Eagle's medium (DMEM) (Gibco, USA) supplemented with 10 % fetal bovine serum (Invitrogen, USA) and incubated at 37 °C in a humidified atmosphere containing 5 % CO2, and subcultured every other day.

Tissue Samples and Tissue Microarray

Tissue specimens and clinical information were obtained as part of an approved study by the Institutional Review Board at the Tianjin Medical University. Forty three human glioma tissues were collected with patient's consent at the time of operation and classified according to 2007 WHO categories. The tissue samples included: 12 grade I–II astrocytomas, 15 grade III anaplastic astrocytomas and 16 grade IV primary glioblastomas. Each tumor tissue samples were taken from three separate viable areas of tumors, five normal brain tissue samples were derived from the temporal lobes of patients surgically treated for intractable temporal lobe epilepsy and astrocytomas verified histologically. Tissue samples for analysis were snap-frozen and stored in liquid nitrogen. All the tumors or NBTs were diagnosed by two independent neuropathologists.

Tissue microarray was prepared by ChaoYing Biotechnology Company (Shanxi, China) from archival paraffin embedded glioma specimens. In each tissue microarray slide, there were 75 cases of astrocytomas, including 10 cases of WHO I, 13 cases of WHO II, 34 cases of WHO III, 18 cases of WHO IV and 5 cases of normal brain tissue.

qRT-PCR Analysis

Total RNA from glioma cell lines and tissues was isolated using Trizol reagent (Invitrogen, USA). For analysis of miRNA expression, real-time RT-PCR analyses were carried out using Hairpin-itTM miRNAs qPCR Quantitation Kit (Shanghai GenePharma Co., Ltd, China) according to the manufacturer's manual. Relative expression was calculated using the $2^{\Delta\Delta CT}$ method [20] and normalized to the expression of U6. All qRT-PCRs were performed in triplicate, and the data were presented as means ± standard errors of the means (SEM).

miR-30a-5p primer	Upper:CACTCTCATGTAAACATCCTCGAC
	Lower:TATGGTTGTTCTGCTCTCTGTGTC
U6snRNA primer	Upper:ATTGGAACGATACAGAGAAGATT
	Lower:GGAACGCTTCACGAATTTG

In Situ Hybridization

Using antisense locked nucleic acid (LNA)-modified oligonucleotides probe, in situ hybridization was performed with In situ hybridization kit (Boster Sci corp, Wuhan, China). The sequence of LNA-miR-30a-5p was: 5'-CTTCCAGTCGAG GATGTTTACA-3'. The level of miR-30a-5p expression in each specimen and glioma cell lines was scored according to the staining intensity: (-) indicated negative expression, (+) indicated weak expression, (++) indicated moderate expression and (+++) indicated strong expression. All samples were anonymized and independently evaluated by two pathologists.

Statistical Analysis

Data were expressed as means ± SEM. Statistics was determined by ANOVA and Spearman correlation test using SPSS11.0. Statistical significance was determined as $P<0.05$ (*) or $P<0.01$ (**).

Results

miR-30a-5p Expression Detected by qRT-PCR

At first,we performed qRT-PCR to assess the mean level of miR-30a-5p in 7 glioma cell lines and 43 glioma samples.

The comparative Ct ($2^{\Delta\Delta Ct}$) method was used to determine the change of miR-30a-5p expression in tumor samples relative to that in normal brain tissue. Briefly, the ΔCt of miRNA in each sample was determined relative to U6 endogenous control RNA, which was robustly and invariantly expressed across all samples, and the average ΔCt of the normal brain tissue samples was used as the calibrator for the tumor samples. MiR-30a-5p was up-regulated two to nine fold in glioma cell lines, i.e. miR-30a-5p expression of malignant glioma cell lines over normal brain tissue was: 7.52±0.12-fold in U251, 8.71±0.30-fold in LN229, 4.47±0.06-fold in SNB19, 2.57±0.15-fold in LN308, 2.54±0.08-fold in U87, 3.15±0.11-fold in TJ899, 4.47±0.06-fold in TJ905 (Fig. 1a, Table 1). miR-30a-5p was also consistently upregulated in our sample set and positively correlated with the tumor grade. MiR-30a-5p expression elevated over control in detail was 3.15±2.76-fold in WHO I~II glioma, 6.78±5.46-fold in WHO III glioma, and 7.71±4.99-fold in WHO IV glioma(Fig. 1b, Table 2).

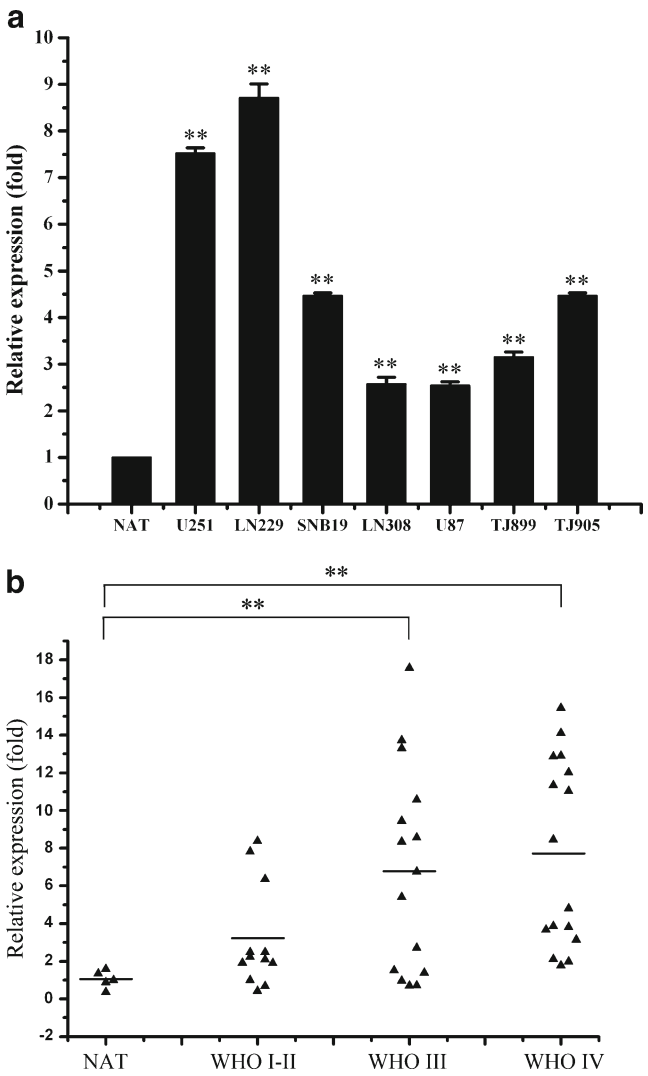

Fig. 1 The miR-30a-5p expression in the gliomas detected by qRT-PCR. **a** MiR-30a-5p overexpression in 7 glioma cell lines. U6 snRNA was used as a loading control. **b** qRT-PCR showed that miR-30a-5p was upregulated in the glioma tissues

Table 1 miR-30a-5p was upregulated in 7 glioma cell lines compared with NBT. The differences of expression level of miR-30a-5p in NBT and glioma cell lines were statistically significant by analysis of variance ($P<0.05$, compared to NBT)

Cell line	Relative fold ($\bar{x} \pm s$)	F value	P value
NBT	1.00±0.00	1.088E3	
U251	7.52±0.12		0.000
LN229	8.71±0.30		0.000
SNB19	4.47±0.06		0.000
LN308	2.57±0.15		0.000
U87	2.54±0.08		0.000
TJ899	3.15±0.11		0.000
TJ905	4.47±0.06		0.000

miR-30a-5p Expression Detected by ISH

MiR-30a-5p was further assayed by ISH for validation, and its expression was normalized with the corresponding U6 RNA in each sample. With fluorescent microscopic examination, miR-30a-5p was barely detected in the normal glial cell cytoplasm, whereas the signals were much stronger detected in the cytoplasm of glioma cell lines (Fig. 2a, Table 3). The similar result was observed in the glioma tissues. MiR-30a-5p overall expression rate in 75 cases of gliomas was 93.3 % (70/75) and 100 % in 52 high grade gliomas. Furthermore, miR-30a-5p displayed various intensity in different grade of gliomas. Its intensity was enhanced with the ascending order of tumor grade. Therefore, miR-30a-5p expression is positively correlated with tumor grade. This result confirmed that we found in qRT-PCR examination(Fig. 2b, Table 4).

Discussion

The role of miRNA in glioblastoma (GBM) has been suggested by Ciafre et al. They studied the global expression of

Table 2 miR-30a-5p was overexpressed in glioma tissues compared with NBT. The differences of expression level of miR-30a-5p in NBT and glioma tissues were statistically significant by analysis of variance ($P<0.05$, compared to NBT)

Grade	Number	Relative fold ($\bar{x} \pm s$)	F value	P value
NBT	5	1.04±0.47	4.502	
WHO I~II	12	3.15±2.76		0.378
WHO III	15	6.78±5.46		0.016
WHO IV	16	7.71±4.99		0.005

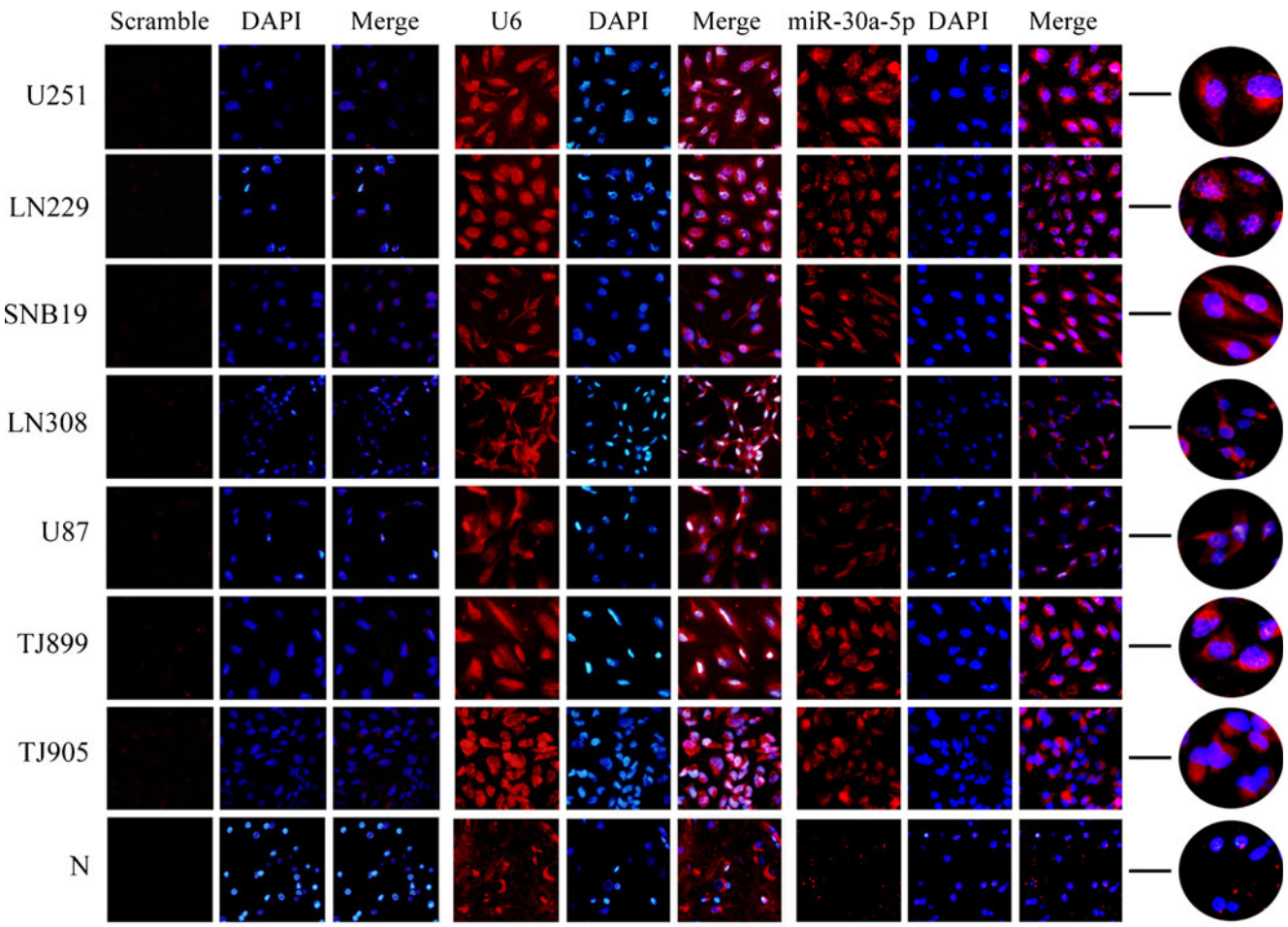

Fig. 2 The miR-30a-5p expression in the gliomas detected by ISH. **a** ISH showed that miR-30a-5p was not expressed or barely detected in normal brain tissues, while it was upregulated in the cytoplasm of glioma cell lines. **b** ISH showed that miR-30a-5p was highly expressed in gliomas, either the percentage of positive staining cells or the intensity of miR-30a-5p expression was increased with tumor grade

245 microRNAs in GBM using a microarray technique [12]. This approach enabled the identification of miRNAs whose expression is significantly altered in tumors compared with nearby normal brain areas from the same patient. They found that nine (miR-221, miR-23a, miR-24-2, miR-24-1, miR-23b, miR-21, miR-222-prec, miR-191, miR-220) and seven miRNAs(miR-181a, miR-181b, miR-128b, miR-197, miR-181c, miR-125b-2, miR-125b-1), respectively, to be up-/down-regulated in human glioblastoma cell lines. Using oligonucleotide arrays specific for 180 human and mouse miRNAs, Chan et al also found marked elevation of five miRNAs (miR-21, miR-138, miR-347, miR-291-5', miR-135) and three miRNAs (miR-198, miR-188, miR-202) to be downregulated in glioblastoma samples [21]. Dong et al examined 470 human miRs in 240 GBM tissue samples and 10 normal brain tissue samples and demonstrated that a total of 97 miRs were significantly differentially expressed in tumor samples as compared with normal brain tissues. Alteration pattern in 22 of these miRs was exactly the same as that reported in published literatures [22]. Several reports described an association between miRNA expression and tumor grade, such as the overexpression of miR 10b, miR 221, miR-17-3p, miR-17-5p, miR-92a-1, miR-106b and downregulation of miR128 were more significant in high grade gliomas [23]. All the studies above suggest a great potential for the use of miRNA profiling as a powerful diagnostic and prognostic marker in defining the signature of astrocytomas, and also implicate that targeting miR may be developed as a novel strategy for the treatment of GBM. However,the involvement of miR-30a-5p in gliomas has not been reported previously.

There have been some reports on the role of miR-30 family members that may be related to the pathogenesis of tumors. Mitochodrial fission is involved in the initiation of apoptosis while apoptosis is relevant to the tumorigenesis. Li et al reported that the overexpression of miR-30 family members, including miR-30a, miR-30b and miR-30d, was able to reduce mitochondrial fission initiated apoptosis by

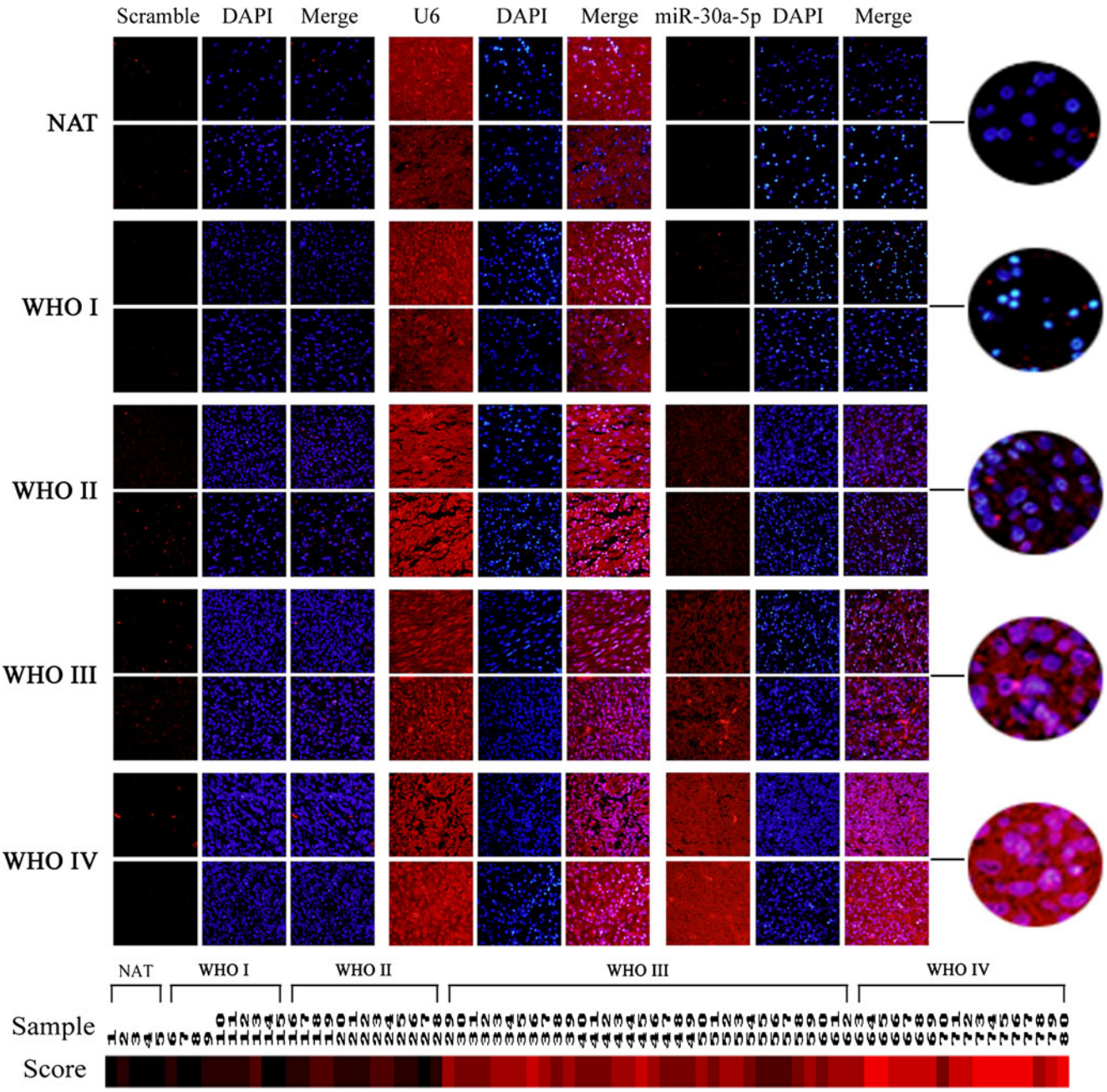

Fig. 2 (continued)

targeting P53, which is transcriptionally activated the mitochondrial fission protein, dynamin-related protein-1 (Drp1) [24]. Cell senescence is a major tumor suppressing mechanism. Martinez et al. demonstrated that miR-29 and miR-30 regulate B-Myb expression by binding to its 3'UTR and suggested that these microRNAs play an important role in Rb-driven cellular senescence [25]. Yu et al showed that miR-30 is reduced in breast tumor initiating cells (BT-ICs). Enforced constitutive expression of miR-30 in BT-ICs inhibited their self-renewal capacity. Similarly, ectopic expression of miR-30 in BT-IC xenografts reduced tumorigenesis and lung metastasis in nonobese diabetic/severe combined immunodeficient mice, whereas blocking miR-30 expression enhanced tumorigenesis and metastasis [26]. Gaziel-Sovran et al [27] carried out a microRNA analysis of human melanoma, and found that miR-30b/30d upregulation correlated with stage, metastatic potential and poor prognosis. Ectopic expression of miR-30b/30d promoted the metastatic behavior of melanoma cells by directly targeting the GalNAc transferase GALNT7. It has also been demonstrated the down-regulation of miR-30a in chronic lymphocytic leukemia (CLL) [28], lung cancer [29], colon cancer [30], and in acute myeloid leukemia

Table 3 miR-30a-5p was upregulated in 7 glioma cell lines compared with NBT

Cell line	Score			
	-	+	++	+++
NBT	*			
U251				*
LN229				*
SNB19				*
LN308			*	
U87			*	
TJ899				*
TJ905				*

(AML) patients with 11q23 translocation (compared to other AML patients) [31]. It is well known that autophagy is activated in tumor cells during chemotherapy and often contributes to chemoresistance of tumor cells. Zou et al [32] reported that miR-30a could sensitize tumor cells to cis-DDP via reducing beclin 1-mediated autophagy and suggested that upregulation of miR-30a level in tumor cells may be an approach to enhance the efficacy of chemotherapy during cancer treatment. Recently, Hahn et al [33] identified that enforced overexpression of miR-30a-5p via a lentivirus vector system into two different colon carcinoma cell lines was able to suppress colon cancer cell growth by targeting denticleless homolog (DTL),which modulates cell cycle via DTL-Tp53-CDKN1A circuit. From the results of the present study and other reports, it was shown that the expression and function of miR may be different in various types of cancers, whether they are dependent on the cellular context should be studied further.

Our results identify for the first time that miR-30a-5p level is markedly elevated in established glioblastoma cell lines and glioma samples and positively correlated with tumor grade. Overexpression of miR 30a-5p may be a biomarker suitable for diagnosis or prediction of glioma malignancy. Accumulated evidences showed that miRs may function as oncogenes or tumor suppressor genes in the tumorigenesis, so that miR-30a-5p overexpression may indicate its oncogenic role in tumorigenesis. Certainly, further studies are needed to fully elucidate the function and mechanism of miR-30a-5p involved in glioma development and progression, and it is possible to be a potential therapeutic target for glioma.

Table 4 The signal intensity of miR-30a-5p expression was increased and associated with the degree of malignancy of tumors. The differences of expression level between the NBT and glioma tissues were statistically significant by analysis of variance ($P<0.05$). There is positive correlation between tumor grade and level of miR-30a-5p expression as analyzed by Spearman correlation test. ($P<0.05$)

Grade	Score				Positive rate	Total	Linear-by-linear association	Pearson chi-square
	-	+	++	+++				
NBT	4	1	0	0	20 %	5		
I~II	5	18	0	0	78.3 %	23	0.000	0.000
III	0	1	31	2	100 %	34		
IV	0	0	3	15	100 %	18		

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