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The use of hsa-miR-21, hsa-miR-181b and hsa-miR-106a as prognostic indicators of astrocytoma

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ABSTRACT

Background: The aberrant expression of microRNAs (miRNAs) is associated with a variety of diseases including cancers. In the present study, the miRNA expression profile was examined in astrocytoma, a malignant and prevalent intracranial tumour in adults.

Methods: We screened the expression profile of 200 miRNAs in a training sample set consisting of 84 astrocytoma samples and 20 normal adjacent tissue (NAT) samples using the method of stem-loop quantitative RT-PCR. The significantly altered miRNAs were validated in another independent sample set consisting of 40 astrocytoma samples and 40 NAT samples. The correlation of the miRNA levels with survival in astrocytoma samples was estimated by performing Kaplan–Meier survival analysis and univariate/multivariate Cox proportional hazard regression analysis.

Results: After a two-phase selection and validation process, seven miRNAs were found to have a significantly different expression profile in astrocytoma samples upon comparison to the NAT samples. Unsupervised clustering analysis further revealed the great potential of the 7-miRNA profile to differentiate between tumours and normal brain tissues. The down-regulation of hsa-miR-137 in astrocytomas was shown to be associated with advanced clinical stages of this disease. Using Kaplan–Meier survival analysis we showed that low expression of hsa-miR-181b or hsa-miR-106a, or high expression of hsa-miR-21 was significantly associated with poor patient survival. Moreover, Cox proportional hazard regression analysis revealed that this prognostic impact was independent of other clinicopathological factors.

Conclusions: Our results suggest a great potential for the use of miRNA profiling as a powerful diagnostic and prognostic marker in defining the signature of astrocytomas and in predicting the post-surgical outcome.

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1. Introduction

Astrocytomas are malignant and prevalent intracranial tumours that comprise the majority of primary central nervous system tumours in adults.[1] In spite of recent improvements in surgical and radiotherapeutic techniques, the prognosis for astrocytoma patients is still very poor.[2,3] Although a number of genetic and molecular lesions have been correlated with astrocytic tumourigenesis,[4–6] a deep understanding of the molecular basis of this tumour is still far away, and the search for novel prognostic or predictive molecular indicators of astrocytoma is still the primary goal with the hopes of improving the clinical management of this tumour.

Recently, the discovery of microRNAs (miRNAs) has opened a new avenue for both the diagnosis and treatment of cancers.[7,8] miRNAs are endogenous non-coding RNAs consisting of 19–24 nucleotides in length that play an important role in the negative regulation of gene expression by base-pairing to complementary sites on the target mRNAs, thus causing a block in the translation or the degradation of the target mRNAs.[9] Presently, accumulating experimental evidence indicates that miRNAs are aberrantly expressed in different tumour types and that they can have a causal role in tumourigenesis.[10–12] Moreover, it has been well demonstrated that miRNAs are correlated with well-defined clinicopathological features and disease outcomes of cancers.[13,14]

In the present study, we investigated the miRNA expression profiles of human astrocytomas by comparing the expression levels of 200 miRNAs from 124 astrocytoma samples to the miRNA expression levels from 60 normal adjacent tissue (NAT) samples using a stem-loop quantitative reverse transcription polymerase chain reaction (qRT-PCR) assay. We observed a widespread variation in the levels of miRNA expression during astrocytic tumourigenesis. Notably, the profile of seven specific miRNAs exhibited great potential as an application for cancer diagnosis. Using the Kaplan–Meier survival analysis and univariate/multivariable statistical models, we showed that low expression levels of hsa-miR-181b or hsa-miR-106a, or high expression levels of hsa-miR-21 are significantly associated with poor survival in patients with astrocytoma. These results indicate that hsa-miR-21, hsa-miR-106a or hsa-miR-181b may have a strong potential to serve as novel prognostic indicators of astrocytoma.

2. Patients and methods

2.1. Patient collection and study design

The present study consisted of 124 patients who underwent surgery to treat astrocytomas at the Third Affiliated Hospital of Suzhou University between 2000 and 2008. Written informed consent was obtained from all patients or their representatives, and the study was approved by the Research Ethics Board of the Third Affiliated Hospital of Suzhou University.

Notably, patients were eligible for inclusion in the study if they had histologically documented astrocytoma and had undergone surgical resection of the tumour. Other eligibility criteria included: (1) the absence of previous cancers or recurrent tumours; (2) the absence of previous chemotherapeutic or radiotherapeutic treatment; (3) the absence of synchronous multiple cancers and (4) the absence of oligodendroglial tumours or mixed tumours. Sixty NAT samples were also analysed and served as controls. The histological typing of the tumours was performed according to the revised World Health Organisation (WHO) classification system. The demographic and clinical features of the patients are listed in Table 1.

To rigorously identify miRNAs that are differentially expressed in astrocytomas compared to normal brain tissues, a two-phase test was designed. A total of 124 astrocytoma samples and 60 NAT samples were enrolled and were assigned to a training set (84 astrocytoma samples versus 20 NAT samples) or to a validation set (40 astrocytoma samples versus 40 NAT samples) according to the tumour resection rate. We first examined the expression levels of 200 miRNAs in the training set. Importantly, only those miRNAs with a mean fold change >2 or <0.5 and a p-value <0.05 were selected. To validate the findings from the training set, the differentially expressed candidates were assessed in the validation set. The miRNAs were validated as an astrocytoma signature only when they fulfilled three criteria: (1) the mean fold change in the expression was >2 or <0.5; (2) the p-value was <0.05 and (3) the variation in the trend of miRNA expression was concordant between the training set and the validation set.

Clinical follow-up examinations were based on periodic visits (every 3 months during the first year, every 6 months during the second year and then once a year thereafter until death). The time to the event was measured from the time of surgery to death, or to the last recorded follow-up visit date for censored patients. The overall mean follow-up period was 34.3 months (range 1–98 months) for the training set and 37.1 months (range 1–92 months) for the validation set.

2.2. Selection of a suitable target for normalisation

An algorithm known as geNorm was used to assess the expression stability of putative normaliser genes.[15] The geNorm determines the most stable reference gene from a set of tested genes in a given sample panel and then calculates a normalisation factor for each sample based on the geometric mean of a user-defined number of reference genes. Before analysis, the raw C_T value of each normalisation candidate was transformed to a quantity as outlined by the authors of geNorm. The selection of the optimal number of stable normalisers was based on geNorm's pairwise variation analysis using a cut-off value of 0.15.

2.3. RNA extraction and qRT-PCR

Tumour and NAT samples were stored in liquid nitrogen until the time of analysis. The total RNA content was extracted from 100 mg of tumour or NAT sample using the Trizol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The RNA molecules were then treated with RNase-free DNase (TaKaRa, Dalian, China) using a standardised protocol.

Assays to quantify the mature miRNA were conducted as described previously.[16,17] Briefly, 1 μg of total RNA was

Table 1 – Summary of the demographic and clinical features of the 124 astrocytoma samples and 60 NAT samples.

Variable	Training set					Validation set					*p*-Value [c]
	Astrocytoma ($n = 84$)		Control ($n = 20$)		*p*-Value [a]	Astrocytoma ($n = 40$)		Control ($n = 40$)		*p*-Value [b]	
	No.	%	No.	%		No.	%	No.	%		
Average age (years)	48.5 ± 12.6		49.1 ± 13.0		0.862 [d]	46.4 ± 16.1		44.2 ± 15.6		0.537 [d]	0.42 [d]
Age (years)					0.892 [e]					0.652 [e]	0.675 [e]
⩽49	49	58.3	12	60		21	52.5	24	60		
>49	35	41.7	8	40		19	47.5	16	40		
Sex					0.788 [e]					0.823 [e]	0.911 [e]
Male	45	53.6	12	60		21	52.5	22	55		
Female	39	46.4	8	40		19	47.5	18	45		
WHO grade											0.971 [e]
Pilocytic astrocytoma (grade I)	6	7.1				3	7.5				
Diffuse astrocytoma (grade II)	26	31				13	32.5				
Anaplastic astrocytoma (grade III)	31	36.9				13	32.5				
Glioblastoma multiforme (grade IV)	21	25				11	27.5				
Tumour resection rate	>90%					65–90%					
Follow-up											
Alive/dead	53/31					19/21					0.147 [e]
Mean survival time (months)	62.3 ± 4.9					53.7 ± 5.7					0.364 [f]

[a] Astrocytoma samples from training set versus control samples from training set.
[b] Astrocytoma samples from validation set versus control samples from validation set.
[c] Astrocytoma samples from training set versus astrocytoma samples from validation set.
[d] Student *t*-test.
[e] Two-sided λ^2 test.
[f] Log-rank test in the Kaplan–Meier survival analysis.

reverse-transcribed to cDNA using AMV reverse transcriptase (TaKaRa, Dalian, China) and looped antisense primers. The mixture was incubated at 16 °C for 15 min, 42 °C for 60 min and 85 °C for 5 min to generate a library of miRNA cDNAs. Real-time PCR was then performed using an Applied Biosystems 7500 Sequence Detection system (Applied Biosystems, Foster City, CA, USA) and a standardised protocol. In each assay, 1 μl of cDNA (1:50 dilution) was used for amplification. The reactions were incubated in a 96-well optical plate at 95 °C for 5 min, followed by 40 cycles consisting of a 15 s interval at 95 °C and a 1-min interval at 60 °C. All reactions were performed in triplicate. After the reactions were completed, the threshold cycle (C_T) values were determined using the default threshold settings. To normalise the expression levels of the target miRNAs, hsa-miR-16, one commonly used housekeeping miRNA, was used as a reference.[18,19] The relative amount of each miRNA was calculated based on the internal control, hsa-miR-16, using the equation $2^{-\Delta C_T}$, in which $\Delta C_T = C_{T\ miRNA} - C_{T\ miR\text{-}16}$. Notably, all primers used for these assays are listed in Supplementary Table 1.

2.4. Statistical analysis

Statistical comparison of the demographic features between the astrocytoma and NAT samples, or between the astrocytoma samples from training set and validation set, was performed by using Student's *t*-test or two-sided λ^2 test. The differences were considered statistically significant at $p < 0.05$.

Moreover, we identified miRNAs whose expression levels were significantly related to patient survival. The survival curves were estimated using the Kaplan–Meier method in SPSS 13.0, and the resulting curves were compared using the log-rank test. We also computed a level of statistical significance for each miRNA based on a univariate Cox proportional hazard regression model in SPSS 13.0. The joint effect of covariables was examined using a multivariate Cox proportional hazard regression model in SPSS 13.0. The differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. Demographic and clinical features of the astrocytoma patients

Tissue samples obtained from 124 astrocytoma patients, ranging from WHO grade I to grade IV, were enrolled into the study. Sixty NAT samples were analysed and served as controls. These samples were assigned to a training set (84 astrocytoma samples versus 20 NAT samples) or to a validation set (40 astrocytoma samples versus 40 NAT samples) according to the tumour resection rate. The demographic and clinical features of the astrocytoma and NAT samples are listed in Table 1. There was no significant difference in demographic factors between the patient samples and the controls, or between the patient samples from training set and validation set. Of the 84 tumour samples from the training set, 6, 26, 31 and 21 samples were diagnosed as pilocytic astrocytoma (WHO grade I), diffuse astrocytoma (WHO grade II), anaplastic astrocytoma (WHO grade III) or glioblastoma multiforme (WHO grade IV), respectively. Of the 40 tumour samples from the validation set, 3, 13, 13 and 11 samples were characterised as pilocytic astrocytoma, diffuse astrocytoma, anaplastic astrocytoma or glioblastoma multiforme, respectively. There was no significant difference in tumour grade between the patient samples from training set and validation set. In general, patients in the training set had undergone more extensive gross-total resection of the tumour (tumour resection rate > 90%) than those patients in the validation set (65% < tumour resection rate < 90%) and therefore had better survival outcomes (mean survival time = 62.3 months, alive/dead = 53/31) than those in the validation set (mean survival time = 53.7 months, alive/dead = 19/21).

3.2. Stability of RNAs shown by qRT-PCR assay

Due to the fact that some tissues were stored in liquid nitrogen for up to 8 years, we must verify that the miRNAs extracted from these tissues were not degraded. Thus, the astrocytoma samples from the training set were divided into four groups: those stored in liquid nitrogen for 1–27 months ($n = 21$), 28–43 months ($n = 21$), 44–68 months ($n = 21$) and 69–98 months ($n = 21$). The expression levels of eight RNAs, including four large molecular weight RNAs (18s rRNA, 28s rRNA, GAPDH and β-actin), a commonly used normaliser (U6 snRNA) and three previously proposed universal reference miRNAs (hsa-miR-16, hsa-miR-191 and hsa-miR-103),[18–20] were measured using qRT-PCR. No statistically significant differences in the C_T values of these RNAs were observed among the different storage times (Fig. 1A). Moreover, the stability of the RNAs from the astrocytoma and from the NAT samples were also characterised using qRT-PCR analysis. Similar C_T values were observed (Fig. 1B), indicating that the RNAs from both tumour and normal tissues are quite stable even when stored for a long period of time. Taken together, our results demonstrated that the RNA extracted from tissues stored in liquid nitrogen are stable and can be used for analysis.

3.3. Selection of a suitable target for normalisation

Proper normalisation is a critical aspect of quantitative gene expression analysis. An algorithm known as geNorm was used to assess the expression stability of eight putative normaliser genes.[15] The geNorm analysis clearly showed that hsa-miR-16 and hsa-miR-191 were highly consistent in their expression levels across 84 astrocytoma tissue samples (Fig. 1C). These miRNAs were statistically superior to the most commonly used reference RNAs, such as U6 snRNA and β-actin (Fig. 1C). In the subsequent experiments, ubiquitously expressed hsa-miR-16 was used as a normalisation control for the stem-loop qRT-PCR assay.

3.4. Aberrant miRNA expression in astrocytoma

To identify the profile of miRNAs as an astrocytoma signature, we first employed a stem-loop qRT-PCR assay to examine the global expression levels of 200 miRNAs in a sample set including 84 astrocytoma samples and 20 NAT samples (training set). In this assay, a relative quantification method was

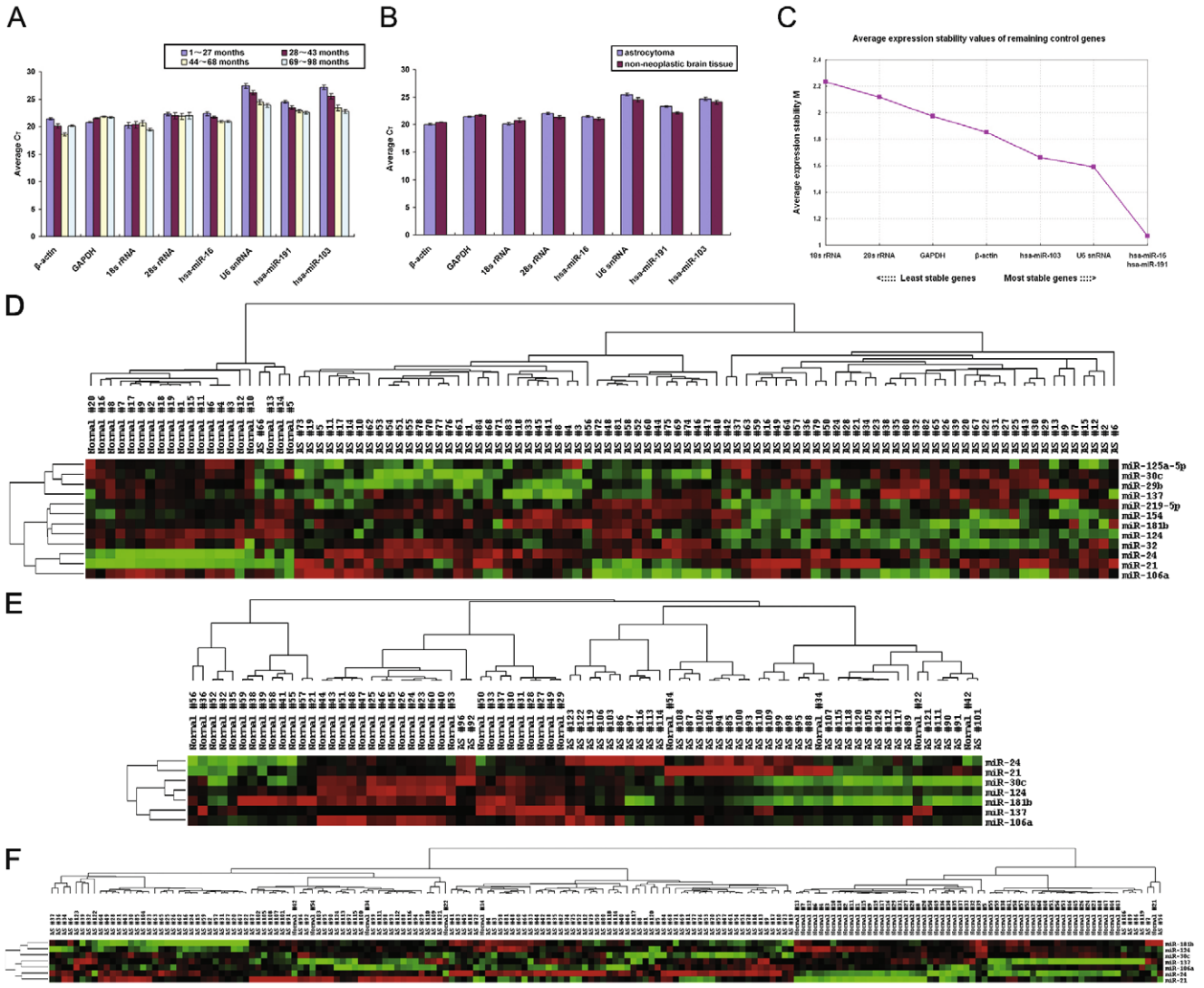

Fig. 1 – Cluster analysis of miRNA that are differentially expressed between astrocytoma samples and normal brain tissues. (A) To investigate the stability of RNAs in astrocytoma samples subjected to long-term storage, the RNAs were isolated from four groups of astrocytoma samples: those stored in liquid nitrogen for 1–27 months ($n = 21$), 28–43 months ($n = 21$), 44–68 months ($n = 21$) and 69–98 months ($n = 21$). The expression levels of eight selected RNAs were measured using qRT-PCR. The C_T values were averaged, and the standard deviation was calculated. (B) RNA was extracted from astrocytoma and NAT samples. The expression levels of eight selected RNAs were measured using qRT-PCR. The C_T values were averaged, and the standard deviation was calculated. (C) The geNorm method ranked the RNA targets from most to least stable. hsa-miR-16 was the most consistently expressed miRNA, followed by hsa-miR-191, U6 snRNA and hsa-miR-103. By contrast, four large molecular weight RNAs, including the 18s rRNA, 28s rRNA, GAPDH and β-actin, were the least stable. (D–F) For training set (D), validation set (E) and all samples (F), the expression levels of miRNAs measured using the qRT-PCR assay were normalised, mean-centred, clustered and plotted as a heat map. The dendrogramme generated by the cluster analysis showed a clear separation of astrocytoma samples from NAT samples based on the 12- or 7-miRNA profiles.

applied. Only those miRNAs with a mean fold change >2 or <0.5 and a p-value <0.05 were selected for further analysis. Based on these parameters, our analysis generated a total of 12 miRNAs that were differentially expressed between astrocytomas and normal brain tissues. The majority of miRNAs exhibiting altered expression levels in astrocytomas showed lower expression levels relative to that observed in normal brain tissues. By contrast, hsa-miR-21 and hsa-miR-24 were up-regulated in astrocytomas. A calculation of the relative amount of these 12 miRNAs is reported in Table 2.

To verify the accuracy and specificity of these 12 miRNAs and refine the number of miRNAs to be used as the astrocytoma signature, we further assessed the 12 miRNAs in another independent sample set consisting of 40 astrocytoma samples and 40 NAT samples (validation set). miRNAs were considered significantly altered only when they fulfilled three criteria: (1) the mean fold change in the expression level was >2 or <0.5; (2) the p-value was <0.05 and (3) the variation in the trend of miRNA expression was concordant between the training set and the validation set. Our analysis ultimately

Table 2 – microRNAs differentially expressed in astrocytoma samples compared to NAT samples.

microRNA	Training set		Validation set		Training + validation		Brain-enriched[24–26]	FRA-associated[23]	Cancer-associated genomic regions[23]
	Mean fold tumour/normal	*p*-Value	Mean fold tumour/normal	*p*-Value	Mean fold tumour/normal	*p*-Value			
hsa-miR-21	7.9075	0.0174	2.5275	0.002983	2.3308	0.003845	No	FRA17B	Amp in breast cancer and neuroblastoma
hsa-miR-24	7.0616	3.4187×10^{-5}	2.2722	0.002845	8.7991	2.333×10^{-8}	No	FRA9D	Del in urothelial cancer and bladder cancer
hsa-miR-29b	0.4687	0.0001031							
hsa-miR-30c	0.4137	2.8123×10^{-8}	0.4163	1.1775×10^{-5}	0.4512	1.6597×10^{-11}	No	FRA9E	
hsa-miR-32	0.4613	0.0005407							
hsa-miR-106a	0.4957	3.6271×10^{-14}	0.2768	8.9084×10^{-5}	0.4402	4.5579×10^{-10}	No		Del in ovarian cancer
hsa-miR-124	0.0841	1.3755×10^{-15}	0.1358	1.0802×10^{-7}	0.1523	4.6079×10^{-8}	Yes		Amp in malignant fibrous histiocytomas
hsa-miR-125a-5p	0.3674	7.7928×10^{-7}							
hsa-miR-137	0.4698	0.01478	0.2108	0.02833	0.4948	0.01062	Yes		
hsa-miR-154	0.4958	0.02609							
hsa-miR-181b	0.4053	0.008687	0.1904	3.5494×10^{-12}	0.4914	0.008426	Yes		
hsa-miR-219-5p	0.4943	0.004949							

Abbreviations: amp, amplification and del, deletion.

Table 3 – Kaplan–Meier survival analysis.

	hsa-miR-21 (Low/high)					hsa-miR-106a (Low/high)			hsa-miR-181b (Low/high)			
	Training set	Validation set	Training + validation	Stage III–IV	⩽46 years	Training set	Validation set	Training + validation	Training set	Validation set	Training + validation	Stage I–III
Mean ± SE (months)	70.8 ± 6.1/ 52.9 ± 7.0	66.6 ± 8.0/ 43.9 ± 7.7	67.9 ± 5.3/ 49.8 ± 5.2	57.8 ± 5.0/ 37.9 ± 6.1	84.3 ± 6.3/ 51.8 ± 6.5	51.1 ± 6.9/ 71.8 ± 6.2	34.6 ± 7.9/ 67.4 ± 6.4	49.6 ± 5.3/ 67.4 ± 5.1	49.3 ± 7.1/ 72.2 ± 6.1	25.1 ± 4.7/ 70.4 ± 5.6	48.3 ± 5.8/ 67.9 ± 4.8	55.4 ± 6.6/ 80.9 ± 4.3
95% Confidence interval (CI) (months)	58.8–82.8/ 39.2–66.5	51.0–82.2/ 28.9–58.9	57.6–78.3/ 39.5–60.0	48.0–67.6/ 25.9–50.0	71.8–96.7/ 39.0–64.6	37.5–64.7/ 59.6–84.1	19.0–50.1/ 54.8–80.0	39.1–60.0/ 57.3–77.4	35.4–63.1/ 60.2–84.2	15.8–34.4/ 59.4–81.4	36.9–59.7/ 58.6–77.2	42.5–68.2/ 72.4–89.4
p-Value	0.061	0.063	0.029	0.01	0.003	0.047	0.005	0.052	0.039	<0.001	0.024	0.008

generated a list of seven miRNAs that were differentially expressed in astrocytomas in comparison with normal brain tissues (Table 2). Among these miRNAs, hsa-miR-21 and hsa-miR-24 were shown to be up-regulated by a factor greater than twofold, whereas five miRNAs, including hsa-miR-106a, hsa-miR-124, hsa-miR-137, hsa-miR-181b and hsa-miR-30c, were shown to be down-regulated by a factor greater than twofold.

The differential expression of miRNAs between astrocytoma tissues and normal brain tissues was further elucidated by an unsupervised clustering analysis that was blind to the clinical annotations. The dendrogramme generated by the cluster analysis showed a clear separation of the astrocytoma samples from the NAT samples based on their respective miRNA profiles (Fig. 1D–F). Of 84 astrocytoma samples and 20 NAT samples from the training set, only one astrocytoma sample was classified incorrectly (Fig. 1D). In the validation set, 40 astrocytoma samples and 40 NAT samples were also clearly separated into two main classes, with two astrocytoma samples and four NAT samples classified incorrectly (Fig. 1E). Finally, a similar result was obtained when we mixed samples from the training set and validation set together, as six astrocytoma samples and five NAT samples were classified incorrectly among 124 astrocytoma samples and 60 NAT samples (Fig. 1F).

3.5. *The association between miRNA expression and clinical outcome in astrocytomas*

We subsequently asked whether the miRNA expression levels represented specific molecular signatures for subsets of astrocytomas. The expression levels of seven miRNAs in 124 tumour samples were stratified using three types of clinical and pathological parameters (gender, age and WHO grade). We performed the student *t*-test to assess the relationship between these clinical features and miRNA expression levels. No miRNAs were identified as being expressed differentially when astrocytoma samples were stratified by age. In addition, hsa-miR-124 was the only miRNA that was expressed differently when the samples were stratified by gender (mean fold change of men/women = 0.37, $p = 0.033$). Furthermore, one miRNA was identified as being differently expressed when the samples were stratified according to the tumour grade. The hsa-miR-137 expression level was lower in grade III/IV astrocytomas when compared to grade I/II astrocytomas (mean fold change = 0.49, $p = 0.02$), as well as in grade I astrocytomas when compared to grade II/III astrocytomas (mean fold change = 0.40, $p = 0.036$). This result suggests that the down-regulation of hsa-miR-137 in astrocytomas is associated with advanced clinical stages of astrocytomas.

3.6. *Correlation between miRNA expression profiles and survival of patients with astrocytoma*

We next investigated the correlation between miRNA expression profiles and the survival outcome using prospective follow-up data collected from 124 astrocytoma patients. Due to the observation that seven miRNAs were expressed differently upon comparison of astrocytomas to normal brain tissues,

Table 4 – Survival analysis of astrocytoma patients in relation to clinicopathological characteristics and miRNA expression.

Variable	Subset	Hazard ratio (95% CI)	*p*-Value
Univariate analysis			
Gender	Female/male	1.067 (0.619–1.84)	0.815
Age	Age ⩾ 47/age < 47	1.926 (1.099–3.373)	0.022
WHO grade	III–IV/I–II	2.096 (1.14–3.851)	0.017
hsa-miR-21	High/low	1.84 (1.051–3.222)	0.033
hsa-miR-106a	Low/high	1.716 (0.985–2.991)	0.057
hsa-miR-181b	Low/high	1.871 (1.072–3.264)	0.027
Multivariate analysis			
Gender	Female/male	1.459 (0.811–2.623)	0.207
Age	Age ⩾ 47/age < 47	1.987 (1.092–3.618)	0.025
WHO grade	III–IV/I–II	1.946 (1.039–3.644)	0.037
hsa-miR-21	High/low	1.882 (1.07–3.308)	0.028
hsa-miR-106a	Low/high	1.629 (0.899–2.954)	0.108
hsa-miR-181b	Low/high	1.862 (1.028–3.372)	0.04

these miRNAs were used for the survival analysis. The expression levels of these seven miRNAs in astrocytomas were first stratified by the median value, then the survival outcome of patients with high miRNA expression levels (⩾median) was compared with that showing low miRNA expression levels (<median) as determined by the Kaplan–Meier survival analysis. We first analysed the follow-up data collected from 84 patients from the training set. We observed a poorer survival rate that was marginally significant in astrocytoma patients showing high levels of hsa-miR-21 ($p = 0.061$; log-rank test) (Supplementary Fig. 1A). The mean survival for patients with high or low levels of hsa-miR-21 was 52.9 months and 70.8 months, respectively (Table 3). By contrast, patients with reduced hsa-miR-106a or hsa-miR-181b expression levels had a poorer survival than patients with high hsa-miR-106a or hsa-miR-181b expression levels, respectively ($p = 0.047$ for hsa-miR-106a and $p = 0.039$ for hsa-miR-181b; log-rank test) (Supplementary Fig. 1B–C, Table 3). To validate the findings from the training set, the three candidate prognostic indicators were assessed using the follow-up data collected from 40 patients from the validation set. We observed a consistent result here (Supplementary Fig. 1D–F, Table 3). More impressively, when we analysed the follow-up data from the training set and the validation set together, all three miRNAs showed a significant correlation with the survival outcomes (Supplementary Fig. 1G–I, Table 3). Taken together, the results suggested that the expression of hsa-miR-21, hsa-miR-181b and hsa-miR-106a following tumour resection may have a prognostic value for astrocytoma patients.

Interestingly, if only grade III/IV astrocytoma patients were enrolled in this study, hsa-miR-21 would have shown an extremely significant correlation with survival outcome ($p = 0.01$; log-rank test) (Supplementary Fig. 1J, Table 3). This result suggests that hsa-miR-21 is more sensitive to predict the clinical outcome of high-grade astrocytomas. By contrast, there was an extremely significant difference for hsa-miR-181b if only grade I/II/III astrocytoma patients were enrolled ($p = 0.008$; log-rank test) (Supplementary Fig. 1K, Table 3). This result suggests that hsa-miR-181b is more sensitive to predict the clinical outcome of low-grade astrocytomas. Furthermore, we noted that hsa-miR-21 showed an extremely significant correlation with survival outcome in younger patients (⩽46 years) ($p = 0.003$; log-rank test) (Supplementary Fig. 1L, Table 3), yet no miRNAs demonstrated a statistically significant correlation with survival outcome in older patients (>46 years). This result suggests that miRNAs are less sensitive in predicting the clinical outcome in older astrocytoma patients.

Subsequently, a univariate Cox proportional hazard regression model was performed to determine the influence of miRNA expression as well as clinicopathological characteristics (gender, age and WHO grade) on patient survival. This univariate analysis indicated that age, WHO grade, hsa-miR-21 expression level and hsa-miR-181b expression level were significantly related to survival, and that the hsa-miR-106a expression level was marginally significantly related to survival, but sex did not influence survival (Table 4). To adjust this potential effect that may be confounded by age, gender and WHO grade, a multivariate Cox proportional hazard regression analysis using all these clinicopathological factors was performed. Upon completing this multivariable analysis, old age, high WHO grade, high hsa-miR-21 expression level and low hsa-miR-181b expression level were all independently associated with decreased survival, while hsa-miR-21 showed a prognostic impact on patient survival as significantly as age (Table 4). These results suggest that hsa-miR-21 and hsa-miR-181b expression levels are important prognostic predictors that are also independent of clinicopathological factors.

4. Discussion

One of the most promising aspects of molecular classification is the possibility of isolating smaller subsets of genes whose expression correlates significantly with clinical parameters in addition to the ability to construct a disease-specific assay that can be used to classify cancers and predict clinical outcome. Currently, it has been well documented that miRNAs can contribute to cancer development and progression.[7,8] miRNAs are differentially expressed in normal tissues and cancer tissues,[10–12] and the abnormal expression of miRNAs in human cancers is significantly associated with the patients' prognosis.[13,14] Thus, the purpose of this study was to

examine the global expression profile of miRNAs in astrocytoma and to investigate its potential relevance to clinicopathological characteristics and patient survival.

By assessing the levels of 200 miRNAs from 124 astrocytoma samples and 60 NAT samples via qRT-PCR, we were able to identify seven miRNAs that were differentially expressed during astrocytic tumourigenesis. Unsupervised clustering analysis further verified that the prediction by using the molecular signature of these seven miRNA is matched to the clinical diagnosis results for these samples. Interestingly, the differentially expressed miRNAs described here, including hsa-miR-21, hsa-miR-124, hsa-miR-137 and hsa-miR-181b, are similar to those listed in previous reports.[21,22] These aforementioned studies, together with our results, firmly support the notion that a miRNA expression profile may generate a unique molecular signature to diagnose astrocytoma.

However, precise molecular mechanisms for the altered expression of miRNAs in astrocytomas remain unclear thus far. One explanation could be that the abnormal expression of miRNAs in astrocytomas was caused by genomic alteration at the abnormal sites on the chromosome referred to as fragile sites.[23] In our data set, five miRNAs (hsa-miR-21, hsa-miR-24, hsa-miR-30c, hsa-miR-106a and hsa-miR-124) were located in these regions. Notably, frequent sister chromatin exchange events, translocations, deletions, amplifications and integrations of exogenous genomic segments among these regions may lead to aberrant miRNA expression as well as tumourigenesis. Thus, the significant differences observed for the expression of miRNAs in astrocytomas appeared to occur, at least partly, as a result of genomic changes. On the other hand, three brain-enriched miRNAs, including hsa-miR-124, hsa-miR-137 and hsa-miR-181b,[24–26] exhibited lower expression levels in astrocytomas relative to normal brain tissues. This result suggests that these miRNAs may play an important role in maintaining differentiation states in normal brain cells and that their down-regulation during tumourigenesis may be a cause of the undifferentiated state of tumour cells.

miRNAs may also have a place in the staging of astrocytomas. When the expression of miRNA was studied in astrocytic tumours of different grades, higher levels of hsa-miR-137 expression were observed in low-grade astrocytomas when compared with high-grade astrocytomas. Interestingly, Silber and colleagues have reported that hsa-miR-137 expression was significantly decreased in high-grade astrocytomas relative to that observed in normal brain tissues and that this can inhibit differentiation of adult mouse neural stem cells in addition to cell cycle arrest of glioblastoma multiforme.[22] Likewise, we also observed lower expression levels of hsa-miR-137 in astrocytomas relative to normal brain tissues and in high-grade astrocytomas relative to low-grade astrocytomas. The aforementioned findings suggest that hsa-miR-137 may serve as a tumour suppressor gene in astrocytoma and that the down-regulation of hsa-miR-137 may play a pivotal role in the initiation and progression of this disease.

The most striking results came from the analysis of hsa-miR-21, hsa-miR-181b and hsa-miR-106a whose altered expression profile was significantly related to the survival of astrocytoma patients. However, the reason that these three miRNAs appear to have a prognostic impact on the survival has yet to be elucidated. This could be due to the anti-apoptotic activities of hsa-miR-21 and/or the anti-proliferative activities of hsa-miR-181b and hsa-miR-106a on tumour biology. It has been well documented that hsa-miR-21 is over-expressed in many human cancers and can function as an anti-apoptotic factor by down-regulating the expression of tumour suppressor genes, such as PTEN and PDCD4.[27,28] By contrast, hsa-miR-181b is known to function as a tumour suppressor gene in human glioma cells.[29] Although hsa-miR-106a is often expressed at a high level in human cancers,[10] a previous report showed that the reduced expression of hsa-miR-106a is associated with poor prognosis in human colon cancer.[30] Our results agree with the observations that hsa-miR-21 may serve as an oncogene and that hsa-miR-181b and hsa-miR-106a may function as tumour suppressors genes during tumourigenesis. It could be speculated that aberrant up-regulation of hsa-miR-21 might block the expression of tumour suppressor genes and that the down-regulation of hsa-miR-181b and hsa-miR-106a might relieve their suppression on oncogenes, which in turn accelerates tumourigenesis. Nevertheless, additional studies to investigate how the altered expression of these miRNAs contribute to the development and/or progression of astrocytomas would improve our understanding of the molecular basis of this tumour, and might ultimately lead to novel therapeutic interventions, as well as diagnostic and prognostic tools for this disease.

In summation, this study represents the initial use of miRNAs to assess their impact on the survival of astrocytoma patients. The capacity of miRNA expression profiles to classify astrocytomas in a manner that is independent of clinicopathological variables highlights the potential application of miRNA signatures as novel prognostic indicators that may contribute to the improved selection of patients for adjuvant therapy.

Conflict of interest statement

None declared.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejca.2010.02.003.

REFERENCES

1. Walker DG, Kaye AH. Diagnosis and management of astrocytomas, oligodendrogliomas and mixed gliomas: a review. *Austral Radiol* 2001;**45**:472–82.

2. Mathieu D, Fortin D. The role of chemotherapy in the treatment of malignant astrocytomas. *Can J Neurol Sci* 2006;**33**:127–40.
3. Okamoto Y, Di Patre PL, Burkhard C, et al. Population-based study on incidence, survival rates, and genetic alterations of low-grade diffuse astrocytomas and oligodendrogliomas. *Acta Neuropathol* 2004;**108**:49–56.
4. Chosdol K, Misra A, Puri S, et al. Frequent loss of heterozygosity and altered expression of the candidate tumor suppressor gene 'FAT' in human astrocytic tumors. *BMC Cancer* 2009;**9**:5.
5. Oji Y, Suzuki T, Nakano Y, et al. Overexpression of the Wilms' tumor gene W T1 in primary astrocytic tumors. *Cancer Sci* 2004;**95**:822–7.
6. Shiraishi S, Tada K, Nakamura H, et al. Influence of p53 mutations on prognosis of patients with glioblastoma. *Cancer* 2002;**95**:249–57.
7. Esquela-Kerscher A, Slack FJ. Oncomirs – microRNAs with a role in cancer. *Nat Rev Cancer* 2006;**6**:259–69.
8. Calin GA, Croce CM. MicroRNA signatures in human cancers. *Nat Rev Cancer* 2006;**6**:857–66.
9. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* 2004;**116**:281–97.
10. Volinia S, Calin GA, Liu CG, et al. A microRNA expression signature of human solid tumors defines cancer gene targets. *Proc Natl Acad Sci USA* 2006;**103**:2257–61.
11. Lu J, Getz G, Miska EA, et al. MicroRNA expression profiles classify human cancers. *Nature* 2005;**435**:834–8.
12. Iorio MV, Ferracin M, Liu CG, et al. MicroRNA gene expression deregulation in human breast cancer. *Cancer Res* 2005;**65**:7065–70.
13. Yanaihara N, Caplen N, Bowman E, et al. Unique microRNA molecular profiles in lung cancer diagnosis and prognosis. *Cancer Cell* 2006;**9**:189–98.
14. Calin GA, Ferracin M, Cimmino A, et al. A MicroRNA signature associated with prognosis and progression in chronic lymphocytic leukemia. *New Engl J Med* 2005;**353**:1793–801.
15. Vandesompele J, De Preter K, Pattyn F, et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol* 2002;**3**:RESEARCH0034.
16. Chen C, Ridzon DA, Broomer AJ, et al. Real-time quantification of microRNAs by stem-loop RT-PCR. *Nucleic Acids Res* 2005;**33**:e179.
17. Tang F, Hajkova P, Barton SC, Lao K, Surani MA. MicroRNA expression profiling of single whole embryonic stem cells. *Nucleic Acids Res* 2006;**34**:e9.
18. Hebert SS, Horre K, Nicolai L, et al. Loss of microRNA cluster miR-29a/b-1 in sporadic Alzheimer's disease correlates with increased BACE1/beta-secretase expression. *Proc Natl Acad Sci USA* 2008;**105**:6415–20.
19. Ben-Ami O, Pencovich N, Lotem J, Levanon D, Groner Y. A regulatory interplay between miR-27a and Runx1 during megakaryopoiesis. *Proc Natl Acad Sci USA* 2009;**106**:238–43.
20. Peltier HJ, Latham GJ. Normalization of microRNA expression levels in quantitative RT-PCR assays: identification of suitable reference RNA targets in normal and cancerous human solid tissues. *RNA* 2008;**14**:844–52.
21. Conti A, Aguennouz M, La Torre D, et al. MiR-21 and 221 upregulation and miR-181b downregulation in human grade II–IV astrocytic tumors. *J Neurooncol* 2009;**93**:325–32.
22. Silber J, Lim DA, Petritsch C, et al. miR-124 and miR-137 inhibit proliferation of glioblastoma multiforme cells and induce differentiation of brain tumor stem cells. *BMC Med* 2008;**6**:14.
23. Calin GA, Sevignani C, Dumitru CD, et al. Human microRNA genes are frequently located at fragile sites and genomic regions involved in cancers. *Proc Natl Acad Sci USA* 2004;**101**:2999–3004.
24. Sempere LF, Freemantle S, Pitha-Rowe I, et al. Expression profiling of mammalian microRNAs uncovers a subset of brain-expressed microRNAs with possible roles in murine and human neuronal differentiation. *Genome Biol* 2004;**5**:R13.
25. Beuvink I, Kolb FA, Budach W, et al. A novel microarray approach reveals new tissue-specific signatures of known and predicted mammalian microRNAs. *Nucleic Acids Res* 2007;**35**:e52.
26. Liang Y, Ridzon D, Wong L, Chen C. Characterization of microRNA expression profiles in normal human tissues. *BMC Genomics* 2007;**8**:166.
27. Meng F, Henson R, Wehbe-Janek H, et al. MicroRNA-21 regulates expression of the PTEN tumor suppressor gene in human hepatocellular cancer. *Gastroenterology* 2007;**133**:647–58.
28. Chen Y, Liu W, Chao T, et al. MicroRNA-21 down-regulates the expression of tumor suppressor PDCD4 in human glioblastoma cell T98G. *Cancer Lett* 2008;**272**:197–205.
29. Shi L, Cheng Z, Zhang J, et al. hsa-mir-181a and hsa-mir-181b Function as tumor suppressors in human glioma cells. *Brain Res* 2008;**1236**:185–93.
30. Diaz R, Silva J, Garcia JM, et al. Deregulated expression of miR-106a predicts survival in human colon cancer patients. *Genes Chromosomes Cancer* 2008;**47**:794–802.