EUROPEAN JOURNAL OF CANCER 47 (2011) 953–963

available at www.sciencedirect.com

ScienceDirect

journal homepage: www.ejconline.com

miR-124a is frequently down-regulated in glioblastoma and is involved in migration and invasion

Adam Fowler [a,b,c,h], *Daniel Thomson* [a,f,i], *Keith Giles* [d,e], *Sanaz Maleki* [a,b], *Ellein Mreich* [f], *Helen Wheeler* [a,g,h], *Peter Leedman* [d,e], *Michael Biggs* [c,h], *Raymond Cook* [c,h], *Nicholas Little* [c,h], *Bruce Robinson* [a,b], *Kerrie McDonald* [a,b,f,*]

[a] *Cerebral Tumour Research Group, Hormones and Cancer, Kolling Institute of Medical Research, NSW, Australia*
[b] *Sydney Medical School, University of Sydney, NSW, Australia*
[c] *Department of Neurosurgery, Royal North Shore Hospital, NSW, Australia*
[d] *Laboratory for Cancer Medicine, Western Australian Institute of Medical Research, WA, Australia*
[e] *University of Western Australia, Australia*
[f] *Prince of Wales Clinical School, Adult Cancer Program, Cure For Life Neuro-Oncology Group, Lowy Cancer Research Centre, University of NSW, Australia*
[g] *Department of Oncology, Royal North Shore Hospital, NSW, Australia*
[h] *Sydney Neuro Oncology Group, Centre for Cancer Biology, Adelaide University, SA, Australia*

ARTICLE INFO

Article history:
Received 7 September 2010
Received in revised form 24 November 2010
Accepted 26 November 2010
Available online 31 December 2010

Keywords:
miR-124a
GBM
Migration
Invasion
Overall survival
IQGAP1
LAMC1
ITGB1

ABSTRACT

Glioblastoma (GBM) represents a formidable clinical challenge for both patients and treating physicians. Due to better local treatments and prolonged patient survival, remote recurrences are increasingly observed, underpinning the importance of targeting tumour migration and attachment. Aberrant expression of microRNA (miRNA) is commonly associated with cancer and loss of miR-124a has previously been implicated to function as a tumour suppressor. The assessment of miR-124a in clinical specimens has been limited and a potential role in migration and invasion has been unexplored until now. We measured the expression levels of mature miR-124a in a retrospective series of 119 cases of histologically confirmed GBM and found its expression was markedly lower in over 80% of the GBM clinical specimens compared to normal brain tissue. The level of reduction in the clinical cohort varied significantly and patients with lower than the average miR-124a expression levels displayed shorter survival times. Endogenous miR-124a expression and the protein expression of three of its targets; IQ motif containing GTPase activating protein 1 (IQGAP1), laminin γ1 (LAMC1) and integrin β1 (ITGB1) were significantly reciprocally associated in the majority of the clinical cases. We confirmed this association in our *in vitro* model. Functionally, the ectopic expression of mature miR-124a in a GBM cell line resulted in significant inhibition of migration and invasion, demonstrating a role for miR-124a in promoting tumour invasiveness. Our results suggest that miR-124a may play a role in GBM migration, and that targeted delivery of miR-124a may be a novel inhibitor of GBM invasion.

* *Corresponding author:* Present address: Prince of Wales Clinical School, Adult Cancer Program, Cure For Life Neuro-Oncology Group, Lowy Cancer Research Centre, University of NSW, Australia. Tel.: +61 293851471; fax: +61 293851510.
E-mail address: k.mcdonald@unsw.edu.au (K. McDonald).
[i] Present address: School of Medicine, Centre for Cancer Biology, Adelaide University, SA, Australia.
0959-8049/$ - see front matter
doi:10.1016/j.ejca.2010.11.026

1. Introduction

Although glioblastoma (GBM) cells rarely metastasize outside the brain, they have an extraordinary capacity to infiltrate the brain parenchyma and migrate long distances from the primary tumour mass. As a result of better local treatments and prolonged survival, recurrences at distances from the initial tumour presentation are increasingly observed, underlying the importance of targeting tumour migration and invasion.

MicroRNAs (miRNAs) are a family of short, endogenous non-coding RNAs that repress gene expression via binding to specific target mRNAs. A single miRNA has the capacity to coordinately regulate a large number of target mRNAs, often belonging to a single signalling pathway. Aberrant miRNA expression and activity have been associated with a range of human diseases, including cancer.[1] Distinct miRNA expression profiles have been associated with GBM, and oncogenic roles have been suggested for miR-21,[2,3] miR-10b[4] and miR-26a whilst up-regulated levels of miR-296 have been associated with angiogenesis.[5] Significant down-regulation of miR-326,[6] miR-128,[7,8] miR-181 and miR-181b,[9] miR-7,[10] miR-137 and miR-124a[11] has also been reported in GBM, suggesting that these miRNAs act as tumour suppressors in this disease. MiR-124a is abundantly expressed in normal brain tissue,[12] necessary for neuronal differentiation[13] and is conserved across species. Embryonic neuronal precursor cells have undetectable expression of miR-124a as they migrate to positions in the cortex and spinal cord. As this migration comes to an end, the expression levels of miR-124a increase during cell differentiation.[12] Transfection of miR-124a into GBM cell lines has resulted in G1 cell cycle arrest through the direct targeting of cyclin dependent kinase 6 (CDK6) suggesting tumour suppressor function.[11] Regulation of CDK6 by miR-124a was also observed in medulloblastoma.[14] No reports have implicated the involvement of miR-124a in tumour migration and invasion.

We examined a large cohort of GBM patients and found that the levels of miR-124a were highly variable and patients with lower levels of miR-124a showed a trend towards poor survival. Our work demonstrates that the alteration of miR-124a is involved in GBM migration and invasion. We also explored the association between miR-124a and three reported mRNA targets, namely IQ motif containing GTPase activating protein 1 (IQGAP1), laminin γ1 (LAMC1) and integrin β1 (ITGB1). These molecules have all been implicated in migration and all have binding sites for miR-124a in the 3′ UTR regions. We show reciprocal expression of miR-124a and all three targets in our GBM cohort. These findings suggest that miR-124a could act as a biomarker in GBM and that its restoration could be a possible therapeutic approach worthy of evaluation in this disease.

2. Materials and methods

2.1. *Glioma cell lines and culture, normal brain RNA and antibodies*

A172, T98G, U87MG, MO59J, MO59K and CCF-STG1 were acquired from the American Type Culture Collection (ATCC) and were cultured according to ATCC guidelines. Human normal total brain RNA was purchased from Ambion for use in all RT-qPCR experiments. Three additional commercial normal brain RNAs were also included in all RT-qPCR experiments, namely: human parietal cortex superior brain total RNA, human orbital frontal cortex brain total RNA, human parietal cortex posterior total RNA (Ambion, Austin, TX). Another 4 normal brain specimens were acquired from consented patients, three obtained during surgery for their primary tumour and one obtained post-mortem.

The following antibodies were used in our studies: mouse anti-human IQGAP1 (1:300 for immunohistochemistry [IHC]; 1:2500 for western analysis, BD Biosciences, Heidelberg, Germany), mouse monoclonal anti-human integrin β1/CD29 (clone 4B7R) (1:500 for IHC; 1:1000 for western

Table 1 – Clinical features of glioblastoma (GBM) cohort grouped according to miR-124a expression.

Characteristic	High miR-124a (above average expression) $n = 53$	Low miR-124a (below average expression) $n = 64$
Age (year)		
Mean	63.86	58.56
Range	(27–84)	(33–85)
Sex-no. (%)		
Male	34 (64)	45 (70)
Female	19 (36)	19 (30)
Extent of surgery-no. (%)		
Biopsy	3 (6)	5 (11)
Debulking	50 (94)	59 (89)
Treatment-no. (%)		
Surgery only	3 (6)	3 (5)
Surgery plus radiotherapy	7 (13)	9 (14)
Surgery, radiotherapy and chemotherapy	43 (81)	52 (81)

Note: clinical data only displayed for 117 of 119 patients tested. Data was incomplete for two patients.

analysis, R&D Systems, Minneapolis, MN), mouse monoclonal anti-human laminin $\gamma 1$ (clone D18) (1:100 for IHC; 1:1000 for western analysis, ABCAM, Cambridge, UK) and rabbit anti-human eif4e (1:1000 for western analysis only, Cell Signalling Technology, Danvers, MA). Normal fraction rabbit IgG (Cell Signalling Technology, Danvers, MA) and mouse IgG_1 (Dako, Glostrup, Denmark) were used as negative controls for IHC.

2.2. Measurement of miR-124a expression and IQGAP1 protein expression in a patient cohort

Human research ethics committee (HREC) approval was obtained for all experiments. A retrospective series of 119 cases of histologically confirmed GBM were selected from Royal North Shore (RNS) and North Shore Private (NSP) Hospitals. Inclusion criteria included (a) age $\geqslant$18 years; (b) had not received any prior therapy for the tumour; (c) had archival formalin fixed paraffin embedded (FFPE) tissue available for microRNA extraction and IHC staining (Table 1).

FFPE tissue (2× 10 μm sections for miRNA extraction, 1×4 μm section mounted on ultra-frost slides for IHC) was obtained from the Department of Anatomical Pathology, RNS. Extraction of miRNA was carried out using RecoverAll™ Total Nucleic Acid kit (Ambion, Austin, TX) according to the manufacturer's recommendations.

MicroRNA quantification of the FFPE extracted RNA was performed using the ABI 7900HT real time PCR system (Applied Biosystems Inc., Foster City, CA). Hsa-miR-124a and RNU48 endogenous control primers and probes (Ambion, Austin, TX) were used with real-time or reverse-transcriptase PCR (RT-PCR) core reagents and Taqman® Universal PCR mastermix (Applied Biosystems Inc., Foster City, CA). ΔΔCt and relative quantification (RQ) values were calculated using Sequence Detection System v2.3 and RQ-manager software (Applied Biosystems Inc., Foster City, CA), and further analysis was performed with SPSS Statistic 18.0 and DataAssist™ v1.0 (Applied Biosystems Inc., Foster City, CA) packages.

2.3. Immunohistochemistry (IHC)

Serial FFPE sections (4 μm) cut from the same paraffin blocks used earlier for miRNA isolation was mounted onto ultra-frost microscope slides. IHC for IQGAP1, LAMC1 and ITGB1 was carried out as previously described.[15] In brief, FFPE sections were de-paraffinised in xylene and re-hydrated through graded ethanols to fresh water, then sections were heat retrieved sections in a boiling waterbath for 20 min using a commercial pH 6.0 citrate buffer (Dako, Glostrup, Denmark) for LAMC1 and ITGB1 and a commercial pH 9.0 EDTA buffer for IQGAP1 and cooled gently, followed by a 3% hydrogen peroxide (3%) quench and digestion by proteinase K. The primary antibodies (concentrations provided above) were added for 30 min at room temperature and were detected using the Dako Envision + Dual Link peroxidase system for 30 min at room temperature with chromagenic visualisation detected with 3,3′-diaminobenzidene (Dako, Glostrup, Denmark). Sections were then counterstained with Harris haematoxylin, dehydrated through graded ethanols, cleared in xylene and mounted. Assays were performed using an automatic platform, Dako Autostainer Plus.

IQGAP1 positivity was assessed by cytoplasmic staining intensity as previously described.[15] Scores of 0–2 were classified as 'negative' whilst scores of 3–4 were classified as 'positive'. Both LAMC1 and ITGB1 staining were heterogeneous in both intensity and frequency. To counter for the stain variability, the Allred methodology of scoring was used. Combined scores of 1–3 were classified 'negative'; scores 4+ were classified 'positive'.

2.4. Locked nucleic acid (LNA)-based in situ hybridisation of microRNA-124a

Deparaffinised FFPE sections were fixed in chilled, 4% paraformaldehyde, washed thoroughly in PBS and then digested with proteinase K (Sigma Aldrich, St. Louis, MO). Sections were pre-hybridised at 30 °C for 2 h and probed overnight with miRCURY™ LNA detection probe for has-miR-124a (3′-DIG (digoxigenin)) (Exiqon®, Vedbaek, Denmark), at an optimised concentration, 20 °C below melting point (*Tm*). Sections were then washed in several changes of increasing stringency buffer (5× SSC, 0.2× SSC) at probing temperature. Sections were blocked in 2% sheep serum solution for 1 h prior to overnight incubation with anti-DIG antibody (1:1000) in a darkened room (20 °C). Sections were then washed several times in developing buffer before addition of colourmetric (NBT/BCIP, Roche®) detection solution at optimised concentration. Incubation was for up to 5 h, at 20 °C and protected from light. Sections were counterstained for nuclear-fast Red and mounted. Visualisation and imaging was performed on an Olympus BX51 microscope with a computer-assisted camera. Sections were scored according to positive or negative status.

Overall survival was determined from the primary surgery date to the date of death or the census date (01/08/2010). Kaplan–Meier survival analysis was used to generate survival curves and estimates of median survival times, with the use of two-sided log rank statistics. Fisher exact test was used to assess associations between miR-124a levels and IQGAP1 expression (IBM SPSS Statistics Version 18.0).

2.5. miRNA precursors and transfections

Synthetic miRNA precursor molecules corresponding to human miR-124a (Ambion, Austin, TX) and the Pre-miR negative control #1 (Ambion, Austin, TX) were used in miRNA-124a transfection experiments. A172 cells were forward-transfected at 80% confluence, using Lipofectamine 2000™ (Invitrogen™Corp., Carlsbad, CA) and miR-124a or negative control precursor molecules at 40 nM final concentration. Cells were maintained in growth media and RNA and protein harvested at 24 and 48 h using Trizol-based (Sigma–Aldrich, St. Louis, MO) and Triton-X-based (Sigma–Aldrich, St. Louis, MO) extraction, respectively.

2.6. RT-qPCR and immunoblotting

Denatured, whole cell lysates were subjected to electrophoresis on NuPage® 4–12% Bis–Tris pre-cast gels (Invitrogen™Corp., Carlsbad, CA) and transferred to nitrocellulose

membrane using a semi-dry method. Chemiluminescence band densitometry was performed using Multi-gauge software (Fujifilm, http://lifescience.fujifilm.com/).

Whole cell RNA was subject to lithium chloride precipitation (Ambion, Austin, TX) with cDNA synthesised by random hexamer conversion. Taqman® gene expression probes (Applied Biosystems Inc., Foster City, CA) for IQGAP1, LAMC1 and ITGB1 were used on Rotorgene 6000 (Qiagen GmbH, Hilden, Germany). Biological and technical replicate data were analysed with response element silencing transcription factor (REST) software (Qiagen GmbH, Hilden, Germany) using a ΔΔCt method. p Values of $\leqslant 0.05$ were considered significant. MicroRNA expression was determined using Taqman® microRNA reverse transcription kit (Applied Biosystems Inc., Foster City, CA) and miR-124a primers and probes as described above.

2.7. Cell proliferation assays

A172 cells were seeded in a 96-well microtiter plate at an optimised density of 5×10^3 cells per well. The number of viable cells in proliferation was determined at 24 and 48 h after transfection using a colorimetric cell proliferation assay (MTS Assay; CelTiter 96 AQU_{EOUS} Non-Radioactive Cell Proliferation Assay; Promega, Madison, WI). Cell proliferation was also assessed with the xCELLigence system (Roche, Basel, Switzerland). Cells were seeded in a disposable E-plate 96 which has incorporated gold cell sensor arrays in the bottom at an optimised density of 5×10^3 cells per well. The E-plate 96 was incubated at 37 °C in a humidified atmosphere containing 5% CO_2. Proliferation of the cells was monitored every 30 min for a period of up to 72 h via the incorporated sensor electrode arrays of the E-Plate 96. The electrical impedance was measured by the RTCA-integrated software of the xCELLigence system as a dimensionless parameter termed cell index (CI).

2.8. Electronic cell-substrate impedance sensing (ECIS), wound healing and invasion assays

To determine the effect of miR-124a on migration, cells were transfected as previously described and plated in 8W10 ECIS culture chips (Applied BioPhysics Inc., Troy, NY). Cells were incubated in growth media for 24 h and then transferred to ECIS equipment (Applied BioPhysics Inc., Troy, NY). Automated wounding was performed at $T + 15$ min and sensing undertaken at 5 Hz. Experiments were performed in triplicate. Data were exported in tabular form and slope calculated for groups over migratory phase. Data were analysed by Student's t-test. p Values $\leqslant 0.05$ were considered significant.

For scratch wounding with miRNA precursors, A172 cells were transfected as previously described and seeded onto six-well culture plates, grown to confluence in normal media for an additional 24 h, at which point the media was changed to DMEM + 1% FBS and wounding was performed with sterile 200 μL pipette tip. At times 0 and 24 h after wounding, phase-contrast images of the healing process were photographed digitally with an Olympus D71 microscope with 20× objective. Three representative images per treatment were analysed and the area closed calculated as a percentage of initial wound area. Groups were analysed by Student's t-test with $p < 0.05$ considered significant.

For invasion assays, Transwell™ six-well plate inserts with 8 μM pore diameter (Corning Inc., Corning, NY) were coated with diluted, growth factor reduced Matrigel™ (BD Biosciences, Heidelberg, Germany) as per manufacturer's recommendation. Lower chambers were filled with growth media and upper chambers with media and 1% FBS. A172 cells were transfected with miRNA precursor molecules and seeded into upper chamber of inserts at 1×10^6 cells per well. After 24 h, inserts were removed, washed and upper matrigel layer was removed a with sterile cotton-swab. The cells having penetrated the membrane and Matrigel™ were fixed in 4% paraformaldehyde (PFA). DAPI nuclear staining was performed and cells counted with Olympus D71 microscope and 20× objective. Experiments were performed in triplicate. Three representative high power field counts per treatment were taken and analysis undertaken by Student's t-test with significance of $p < 0.05$.

3. Results

3.1. MiR-124a is down-regulated in GBM and associated with shorter overall survival

The expression of mature miR-124a was measured in a series of 119 primary GBM patient samples and 6 GBM cell lines (A172, T98G, U87MG, MO59K, MO59J and CCF-STGG) by quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) analysis (Fig. 1). When compared to the mean expression level of 4 normal commercially acquired brain tissue samples, all 6 cell lines demonstrated significant down-regulation of miR-124a transcript ($p \leqslant 0.001$) (Fig. 1A). Large variability in miR-124a transcript levels was observed within the patient sample cohort ($n = 119$) when compared to the mean expression level of 8 normal brain specimens (4 commercial, 4 acquired locally with human ethics approval). Although 15 patients showed higher expression of miR-124a compared to normal brain, the vast majority (87%) displayed reduced miR-124a expression by 10–7800-fold ($p \leqslant 0.001$) (Fig. 1B). The correlation between miR-124a expression and overall survival was measured through Kaplan–Meier survival curve analysis with a log rank comparison using a binomial variable of high or low expression relative to the average expression level (relative quantification; RQ) of miR-124a (Fig. 1C). Survival data were available for 117 of 119 patients. Approximately 52 (44%) of patients showed higher than average miR-124a expression. The median survival of patients with high miR-124a was 13.9 months (95% CI: 12.1–15.6) compared with patients with low miR-124a expression levels (median survival: 13.3 months; 95% CI: 11.9–14.7). The difference in median survival was not significant by log rank comparison ($p = 0.481$). At 2 years, similar survival rates were observed in both groups (19% [$n = 10$] of patients with high and 14% [$n = 9$] of patients with low miR-124a expression). Increasing age and treatment (surgery only, surgery plus radiotherapy and surgery plus radiotherapy and chemotherapy) were independent prognostic factors for survival.

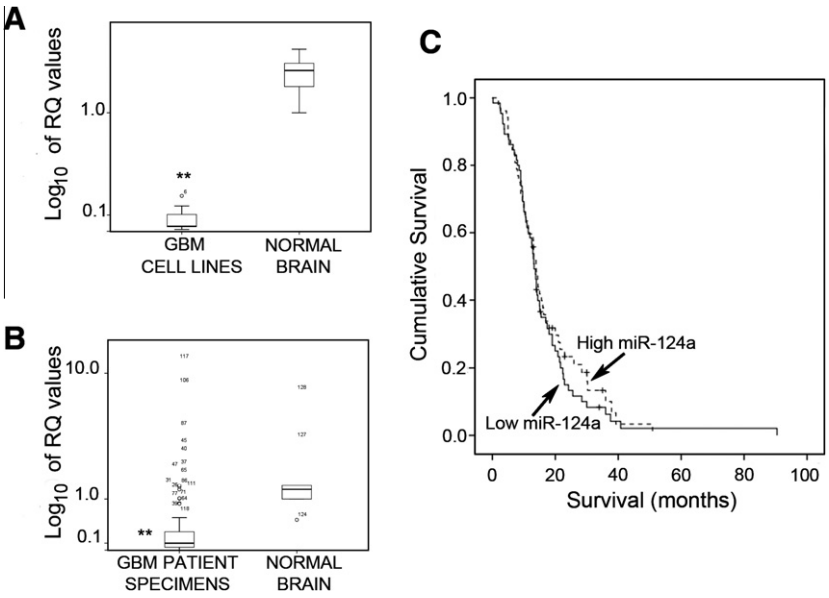

Fig. 1 – The expression of miR124a in glioblastoma (GBM) and its association with survival. Simple box plots indicate $\log_{10}$ of the relative quantification (RQ) values of miR-124a expression levels, normalised by RNU48 for GBM cell lines ($n = 5$) (A) and GBM tumour specimens ($n = 119$) (B) compared to normal brain ($n = 8$). Survival data was available for 117 of the 119 patients. Those patients with high miR-124a (higher than the average RQ values in GBM patients; $n = 52$) showed a slight overall survival benefit compared to patients with low miR-124a (lower than the average RQ values; $n = 65$) (C) (denotes p-value $\leqslant 0.005$).**

3.2. *MiR-124a affects migration and invasion in a GBM cell line*

To analyse the functional role of miR-124a in GBM, we restored miR-124a expression in A172 cells by transient transfection. To confirm miR-124a over-expression in A172 cells, a DIG-labelled, LNA based *in situ* hybridisation method was used (Fig. 2A) and further verified with qPCR (data not shown). Changes in cell proliferation were assessed at 24 and 48 h post-transfection using two independent methodologies (MTS cytotoxicity assay and xCELLigence® live cell analysis). A172 cells transfected with miR-124a (pre-miR-124a; 40 nM) and miRNA negative control 1 (NC-1; 40 nM) showed cell proliferation rates comparable to that of mock-transfected cells (Fig. 2Bi–iv). The concentration of miR-124a was increased to 100 nM to determine if a higher concentration could induce cell death. No significant difference in cell proliferation was observed in A172 cells transfected with miR-124a when compared to cells transfected with NC-1 (100 nM) and mock transfected cells after 24 or 48 h (Supplementary Fig. 1). Cell proliferation was assessed for an additional 24 h (72 h post-transfection) however no difference in any of the treatments was measured.

To test whether miR-124a regulates tumour cell migration the motility of transfected A172 GBM cells was assessed. All experiments were carried out in the presence of serum. Preliminary experiments showed no significant difference in GBM adhesion when cultured on a vitronectin-coated surface compared to serum. Approximately 12 h post-wound scraping of a confluent monolayer, nearly full wound closure was observed in the control cells (NC-1 and mock transfected control) (Fig. 2Ci). In contrast, wounds scraped in confluent cultures of miR-124a transfected cells closed only partially. Electrical cell substrate impedance sensing (ECIS) assays were used to quantitate migration. Using this system, a wound is generated by applying a voltage to the wells. The cells ability to migrate over this wound can be measured in real time based on the impedance changes generated as a result of disruption to the monolayer of cells. The electric resistance changes were recorded and the average of 3 ECIS experiments graphically illustrated in Fig. 2Cii. All A172 control cells (NC-1 and mock transfected control) demonstrated an increase in resistance within 2 h of voltage-mediated wounding and stabilised at approximately 15–25 Ω/h for the duration of the experiment (12 h). A172 cells transfected with miR-124a did not demonstrate this increase in resistance, reflecting a 66% impaired capacity to migrate ($p = 0.009$).

To measure A172 invasion after transfection, matrigel Transwell™ assays were conducted using a serum gradient. Following transfection of miR-124a the number of invasive cells able to digest the collagen and migrate through pores in the membrane was reduced by over 50% ($p = 0.048$) in A172 cells compared to all controls (NC-1, mock transfected and untransfected cells) (Fig. 2Di and ii).

3.3. *MiR-124a targets*

Over 150 gene transcripts were reported to be down-regulated when HeLa cells were transfected with miR-124a.[16] Of particular interest, two putative miR-124a seed sequences in the 3′UTR of IQGAP1 with high complimentarity to miR-124a were identified and the interaction was validated experimentally by the authors.[16] Aberrant expression of IQGAP1 has been implicated as a key mediator of cell migration and invasion

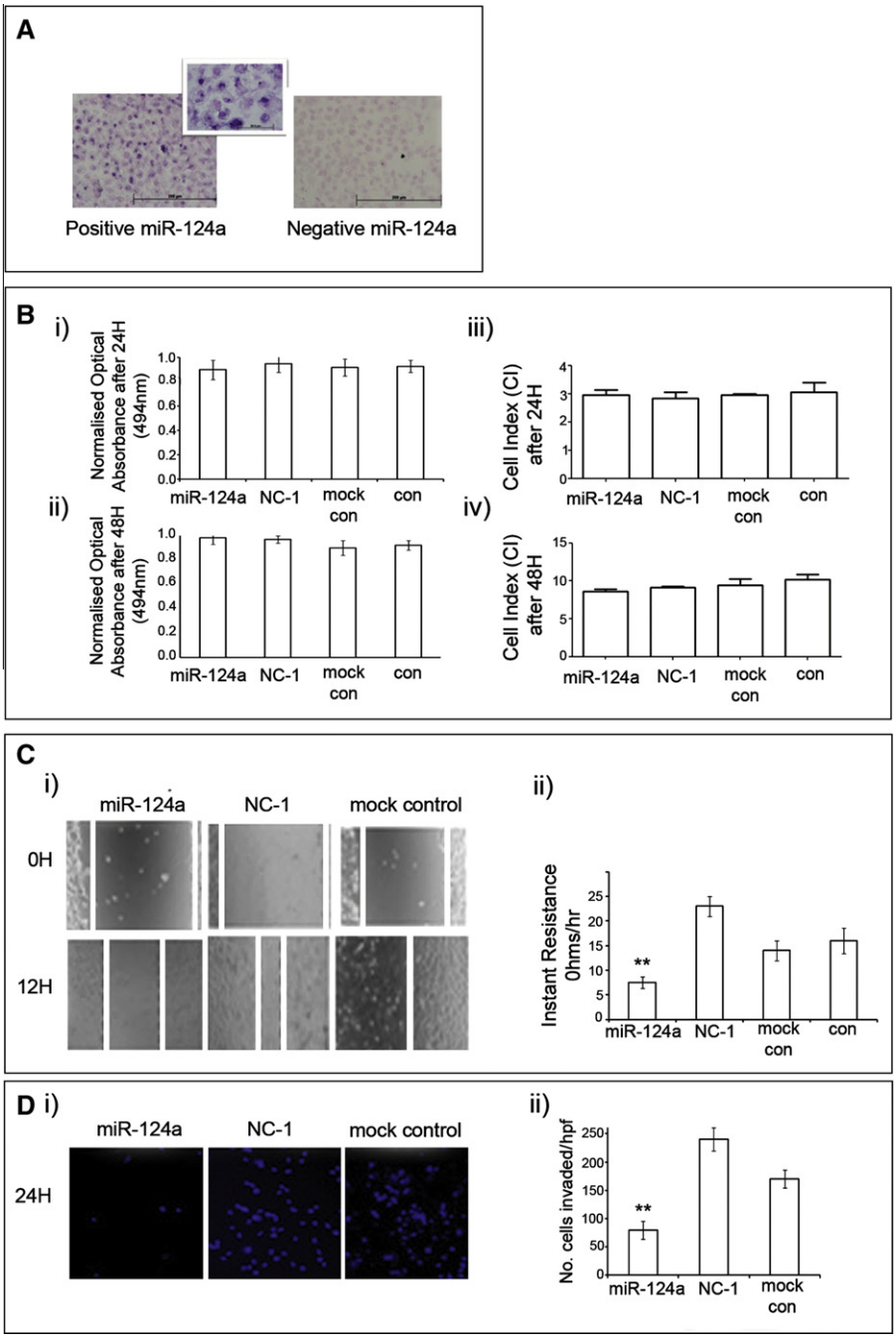

Fig. 2 – Ectopic expression of miR-124a in A172 GBM cells. A172 cells were transfected with commercial precursor miR-124a (miR-124a) molecule (40 nM), pre-miR negative control-1 (NC-1) (40 nM), mock transfected with lipofectamine (mock con) or untransfected (con). Cell pellets were formed and paraffanised. Positive transfection for miR-124a was visualised using DIG-labelled, locked nucleic acid (LNA) based, *in situ* hybridisation (A). Heavy, blue cytoplasmic and nuclear staining can be seen at low power and high power (inset). Cells transfected with NC-1 were absent of this miR-124a staining. Tumour cell growth potential were measured at 24 and 48 h post-transfection (B) with the MTS cytotoxicity assay (i and ii) or xCELLigence cell index (CI) impedance measurements (iii and iv), respectively. Cell migration rates (C) were measured using scratch wound assays (i) and with instantaneous impedance electronic cell-substrate impedance sensing (ECIS) (12 h post-transfection) (ii). The dotted line (shown in i) denotes migratory cellular fronts. Thin-coat Matrigel™ transwell invasion assays were used to measure invasion (D). Transfected and untransfected cells were seeded into Matrigel-coated, 8 μm transwell inserts over serum gradient. Membrane invasion measured at 24 h using DAPI filter microscopy (20×) (i) and the average of three representative high power field (hpf) counts (ii) (denotes *p*-value ⩽0.005).**

in several invasive cancers and elevated levels of IQGAP1 protein in 84% of high grade gliomas were also highly predictive of poor survival outcome.[15] Two additional targets of miR-124a identified with reported roles in GBM migration were LAMC1 and ITGB1.[17] We examined whether IQGAP1, LAMC1 and ITGB1 could be reciprocally regulated by miR-124a both *in vitro* and in our clinical cohort.

IHC for IQGAP1, LAMC1 and ITGB1 were performed on the same clinical specimens used to measure mature miR-124a expression. Representative photos are shown in Fig. 3A. Over-expression of IQGAP1, LAMC1 and ITGB1 was observed in 47%, 77% and 69% of patients, respectively. Strongly positive immunostaining of both IQGAP1 and LAMC1 proteins was evident in the pseudopalisading cells surrounding the central necrotic focus. This staining pattern is clearly evident in the microphotograph of LAMC1 displayed in Fig. 3A. More diffuse, generalised cytoplasmic immunostaining of LAMC1 and IQGAP1 were also evident throughout the tumour sections. The distribution of ITGB1 immunostaining was patchy throughout the tumour sample. Strongly positive areas were often close to the necrotic regions however unlike IQGAP1 and LAMC1, the immunostaining of ITGB1 was not specific to the pseudopalisading cells.

As described earlier, total microRNA was extracted from the same patient FFPE block used for IHC. These GBM specimens were screened with DIG-labelled, LNA based *in situ* hybridisation. MiR-124a expression was not detected in any of the specimens. We believe that this was a sensitivity issue. To obtain a correlation between miR-124a and its target mRNA within the same patient sample, RQ values derived from qPCR were assessed. IQGAP1 protein expression and miR-124a expression were significantly reciprocally associated (two-sided linear by linear association; $p = 0.010$; Fig. 3B). A trend for lower levels of miR-124a associated with high LAMC1 and ITGB1 protein expressions was also observed, although these did not reach statistical significance ($p = 0.056$ and 0.064, respectively; Fig. 3B).

To investigate the miR-124a-target relationships *in vitro*, we transfected A172 cells with miR-124a and evaluated the transcript levels of the IQGAP1, LAMC1 and ITGB1 genes by qRT-PCR at 24 h post-transfection (Fig. 4A). Significantly diminished expression of all mRNA transcripts was observed at 24 h after miR-124a transfection. To test whether miR-124a could regulate protein expression of IQGAP1, LAMC1 and ITGB1, we performed Western blot analysis on cells transfected with miR-124a. Our results showed that the protein expression of all three targets was significantly reduced after 24 and 48 h when compared with control cells (Fig. 4B). Transfection of miR-124a resulted in a threefold reduction in IQGAP1 protein expression whilst the expressions of LAMC1 and ITGB1 were reduced by more than half.

4. Discussion

Loss of miR-124a has been previously described for GBM, however examination of miR-124a in large clinical cohorts has been lacking. Our strategy was to analyse mature miR-124a expression in a large clinical cohort of GBM patients and to consider the association of miR-124a expression with overall survival. Ectopic expression of miR-124a in A172 GBM cells enabled us to address potential functional roles for miR-124a whilst we had the significant advantage of measuring key target proteins using serial sections from the same patient tested for miR-124a expression. Although no significant survival benefits were observed, patients with lower than the average miR-124a expression showed shorter survival

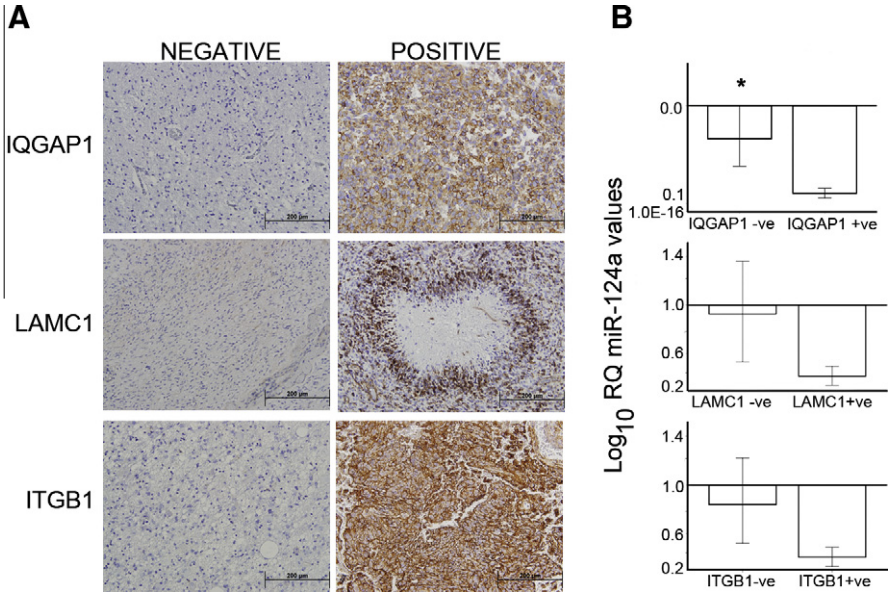

Fig. 3 – Protein expression and association with miR-124a in serial sections from 116 GBM specimens. Representative micrographs of negative and positive immunohistochemistry staining for IQ motif containing GTPase activating protein 1 (IQGAP1), laminin γ1 (LAMC1) and integrin β1 (ITGB1) are shown in (A). All micrographs were taken at 20× magnification. Patients were dichotomised into positive and negative (according to positive or negative target protein expression) and correlation with the levels of miR-124a in each group was assessed (B) (*denotes *p*-value ⩽0.01).

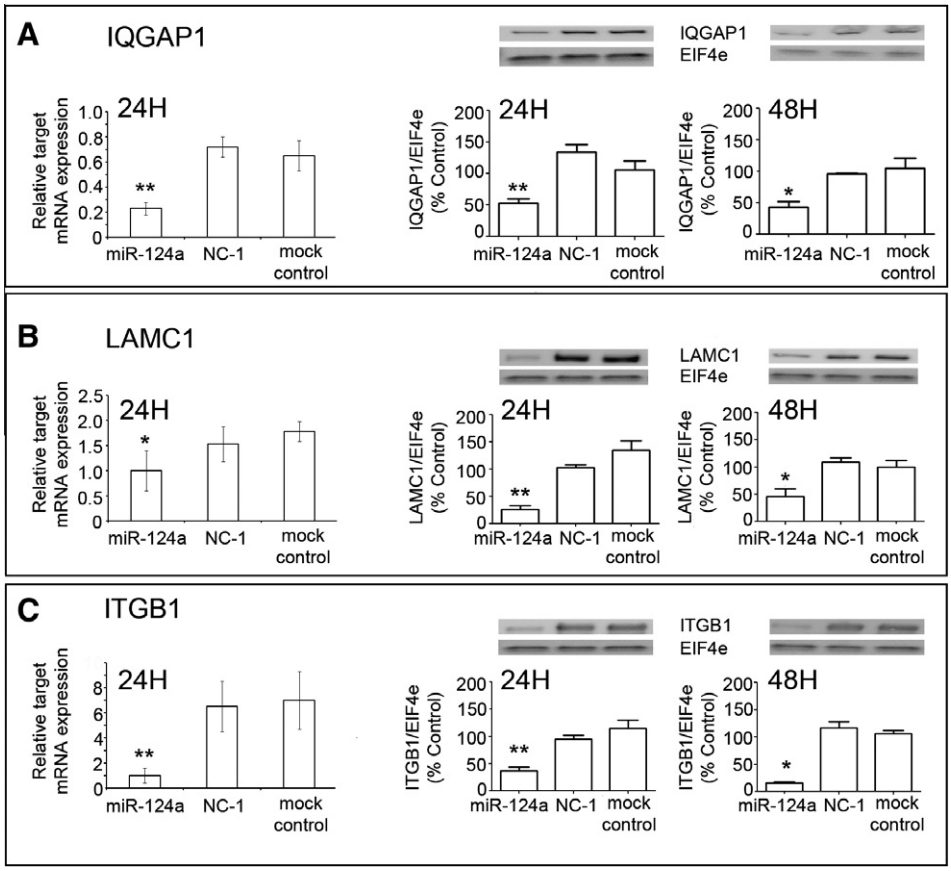

Fig. 4 – Relative expression of IQGAP1, LAMC1 and ITGB1 in GBM A172 cells after transfection with miR-124a. A172 cells were transfected with commercial precursor miR-124a (miR-124a) molecule (40 nM), pre-miR negative control-1 (NC-1) (40 nM) or mock transfected with lipofectamine (mock control). Relative mRNA expression for IQGAP1, LAMC1 and ITGB1 were measured 24 h post-transfection with quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) (A). Relative protein expression for IQGAP1, LAMC1 and ITGB1 were measured 24 h and 48 h post-transfection with Western blot analysis (B). Corresponding immunoblots are shown above the relative quantification of protein signal standardised to the loading control (EIFe4). Each experiment was performed in triplicate (denotes *p*-value ⩽0.005; *denotes *p*-value ⩽0.01).**

times and generally higher expression of three miR-124a protein targets, namely IQGAP1, LAMC1 and ITGB1. This reciprocal association between miR-124a and IQGAP1, LAMC1 and ITGB1 was confirmed in our *in vitro* model. By forcing high miR-124a expression, significant down-regulation of IQGAP1, LAMC1 and ITGB1 at the mRNA and protein expression levels was measured. High miR-124a expression also led to significant inhibition of cell migration and invasion, providing evidence for the first time to our knowledge implicating a role for miR-124a in GBM invasiveness.

Our expression analysis revealed that miR-124a was significantly down-regulated in 87% of GBM. Loss of miR-124a transcript in GBM, oligodendroglioma and also medulloblastoma has previously been reported.[18–20] In a microRNA profiling experiment of GBM tissue compared to normal adjacent brain, miR-124a down regulation was the most significant event.[7] The current study extended these findings by examining the relationship between miR-124a loss and survival for the first time in a large clinical cohort of GBM patients. Significant differences in median overall survival and 2-year survival were not observed when we grouped the patients binomially according to high or low expression relative to the average expression level of miR-124a. Whilst we did not demonstrate a prognostic role for miR-124a loss in GBM, low miR-124a expression levels have been shown to be a marker of increased risk for developing cervical cancer[21] and was associated with a higher relapse rate and mortality rate in patients with acute lymphoblastic leukaemia (ALL).[22]

miR-124a has been widely reported to function as a tumour suppressor.[3,11,23] Ectopic expression of miR-124a in medulloblastoma cells significantly inhibited cell proliferation and blocked cell cycle progression[3] whilst increased expression of miR-124a in a different medulloblastoma cell model inhibited proliferation but not apoptosis.[14] In U251 GBM cells and CD133+ positive GBM-derived cells, G0/G1 cell cycle arrest was induced after over-expressing miR-124a.[11] Transfection of A172 GBM cells with a synthetic miR-124a precursor did not induce any detectable changes in cell proliferation when compared to cells transfected with a negative control-1 (NC-1) or mock transfected. The concentrations of miR-124a and the time-points of analysis were comparable to a previous study who reported anti-proliferative properties.[11] To resolve the discrepant findings, an *in vivo* experiment of implanting GBM cells expressing miR-124a into the

flank of athymic mice to determine if tumour growth is significantly decreased is needed. Neither the current study nor the study reported by Silber and colleagues conducted this *in vivo* experiment.[11]

When we over-expressed miR124a in A172 GBM cells, we observed a significant inhibition of cell migration and invasion. GBM migration was inhibited by approximately 60% whilst invasion in the same model was reduced by 50%. Amongst the hundreds of potential human genes as targets of miR-124a, we chose to examine IQGAP1, LAMC1 and ITGB1. All three targets play a role in GBM migration and invasion. In addition, a biochemical method for identifying miRNA targets that combined RNA-induced silencing complex (RISC) purification with a microarray analysis of bound mRNAs selected IQGAP1, LAMC1 and ITGB1 as direct targets of miR-124a.[24] Overexpression of miR-124a in A172 cells significantly reduced the mRNA and protein expression of IQGAP1, LAMC1 and ITGB1. We further showed evidence of this reciprocal association between miR-124a and all three targets in our clinical cohort of 119 patients.

Increased expression and altered localisation of IQGAP1 has been frequently observed in immortalised cancer cell lines and in various primary neoplasms[25–27] and is a key regulator of cell adhesion, migration and cell polarity acting through the cytoskeleton components.[25,26,28] The migratory cell activities in human embryonic stem cells (hESCs) were shown to be governed by miR-124a, acting through the proteins SLUG and IQGAP1.[29] Cao and colleagues identified LAMC1 and ITGB1 as specific endogenous targets of miR-124a in neural progenitor cells.[13] Laminin is a major structural component of the basement membrane, and is known to promote cell adhesion and migration along with other extracellular matrix (ECM) proteins such as type IV collagen, perlecan and nidogen.[30] Laminins bind to integrins which are also critical for cell migration and invasion. Integrins mediate the interactions between cells and the extracellular matrix (ECM) and regulate the signalling pathways that control cytoskeletal organisation.[31] Recent work in neural stem cells (NSCs) has revealed that LAMC1 functions as part of a ternary protein complex netrin-4/LAMC1/α6β1 integrin to activate the mitogen-activated protein (MAP) kinase signalling pathway, which results in cell migration and proliferation.[32]

We previously reported high levels of IQGAP1 protein to be prognostic of adverse survival in high grade gliomas.[15] We also noted a distribution of IQGAP1 immunostaining specific to the pseudopalisading cells surrounding the necrotic foci.[15] Two other endogenous targets of miR-124a, LAMC1 and ITGB1, were also over-expressed in the majority of GBM patients in our cohort. Experimental evidence of LAMC1 and ITGB1 as miR-124a targets is evident in varied published high-throughput screening methods.[16,17,24,29,33,34] Similar to IQGAP1, immunostaining of LAMC1 was observed to be particularly intense in the pseudopalisading cells, with strongly positive ITGB1 immunostaining often close to the necrotic regions. Areas of pseudopalisading necrosis signify accelerated growth and aggressive tumour behaviour. The pseudopalisading cells also typically express high levels of vascular endothelial growth factor (VEGF), hypoxia inducible factor 1 alpha (HIF1α) and the chemokine, CXCL12 contributing to an outward migration of tumour cells.[35,36] Future work to isolate pseudopalisading cells from the tumour mass and to quantify the precise expression levels of miR-124a and the target proteins will be needed to determine if this reciprocal relationship is specifically contributing to increased migration in the pseudopalisades.

We have not attempted to address the mechanisms underlying miR-124a down-regulation in this study. It has been suggested that miR-124a is suppressed as a result of altered growth factor receptor signalling, such as via epidermal growth factor receptor (EGFR) or vascular endothelial growth factor receptor (VEGFR).[11] Studies examining the potential mechanism of loss of miR-124a expression in hepatocellular cancer concluded a prominent role for epigenetic modification and subsequent miR-124a down-regulation. Supporting this finding, increased miR-124a expression was observed after treating the breast cancer cell line, MCF-7, and the colon cancer line, HCT-116, with DNA de-methylating agents.[39,40] However, similar experiments using U87 and U251 glioma cells treated with demethylating agents did not reveal changes in miR-124a expression,[11,37,38] and thus the precise mechanism of miR-124a down-regulation in GBM remains unclear. Studies examining medulloblastoma have provided strong evidence that miR-124a is under direct regulation by the transcription factor, response element silencing transcription factor (REST).[37,38] This mechanism has not been extensively explored in GBM.

In conclusion, this study has confirmed that loss of miR-124a expression is a common event in GBM and we have provided evidence that miR-124a plays a role in regulating migration and invasion. MiR-124a has multiple predicted conserved targets in the human genome and our data demonstrate that IQGAP1, LAMC1 and ITGB1 are major targets of miR-124a in GBM. With its regulation of glioma migration coupled with increasing evidence supporting a role for miR-124a in the promotion of neuronal differentiation, restoration of miR-124a expression may represent a novel therapeutic strategy in multi-modal GBM therapy.

Conflict of interest statement

None declared.

Acknowledgments

The authors would like to acknowledge the financial support from the Cancer Council of NSW, Andrew Olle Memorial Trust and Sydney Neuro-oncology Group. Dr. Fowler is sponsored by NHMRC, Royal Australasian College of Surgeons, Synthes and Neurosurgical Society of Australasia. Dr. McDonald is supported by a Cancer Institute NSW Career Development and Support Fellowship. The authors would like to express their sincere thankyou to Mrs. Sally Fielding for the rigorous collection of clinical data.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejca.2010.11.026.

REFERENCES

1. Croce CM. Causes and consequences of microRNA dysregulation in cancer. *Nat Rev Genet* 2009;**10**(10):704–14.
2. 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**(3):325–32.
3. Li J, Huang H, Sun L, et al. MiR-21 indicates poor prognosis in tongue squamous cell carcinomas as an apoptosis inhibitor. *Clin Cancer Res* 2009;**15**(12):3998–4008.
4. Sasayama T, Nishihara M, Kondoh T, Hosoda K, Kohmura E. MicroRNA-10b is overexpressed in malignant glioma and associated with tumor invasive factors, uPAR and RhoC. *Int J Cancer* 2009;**125**(6):1407–13.
5. Wang S, Olson EN. AngiomiRs – key regulators of angiogenesis. *Curr Opin Genet Dev* 2009;**19**(3):205–11.
6. Kefas B, Comeau L, Floyd DH, et al. The neuronal microRNA miR-326 acts in a feedback loop with notch and has therapeutic potential against brain tumors. *J Neurosci* 2009;**29**(48):15161–8.
7. Godlewski J, Nowicki MO, Bronisz A, et al. Targeting of the Bmi-1 oncogene/stem cell renewal factor by microRNA-128 inhibits glioma proliferation and self-renewal. *Cancer Res* 2008;**68**(22):9125–30.
8. Zhang Y, Chao T, Li R, et al. MicroRNA-128 inhibits glioma cells proliferation by targeting transcription factor E2F3a. *J Mol Med* 2009;**87**(1):43–51.
9. 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.
10. Webster RJ, Giles KM, Price KJ, et al. Regulation of epidermal growth factor receptor signaling in human cancer cells by microRNA-7. *J Biol Chem* 2009;**284**(9):5731–41.
11. 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.
12. Cheng LC, Pastrana E, Tavazoie M, Doetsch F. MiR-124 regulates adult neurogenesis in the subventricular zone stem cell niche. *Nat Neurosci* 2009;**12**(4):399–408.
13. Cao X, Pfaff SL, Gage FH. A functional study of miR-124 in the developing neural tube. *Genes Dev* 2007;**21**(5):531–6.
14. Pierson J, Hostager B, Fan R, Vibhakar R. Regulation of cyclin dependent kinase 6 by microRNA 124 in medulloblastoma. *J Neurooncol* 2008;**90**(1):1–7.
15. McDonald KL, O'Sullivan MG, Parkinson JF, et al. IQGAP1 and IGFBP2: valuable biomarkers for determining prognosis in glioma patients. *J Neuropathol Exp Neurol* 2007;**66**(5):405–17.
16. Lim LP, Lau NC, Garrett-Engele P, et al. Microarray analysis shows that some microRNAs downregulate large numbers of target mRNAs. *Nature* 2005;**433**(7027):769–73.
17. Chi SW, Zang JB, Mele A, Darnell RB. Argonaute HITS-CLIP decodes microRNA-mRNA interaction maps. *Nature* 2009;**460**(7254):479–86.
18. Gaur A, Jewell DA, Liang Y, et al. Characterization of microRNA expression levels and their biological correlates in human cancer cell lines. *Cancer Res* 2007;**67**(6):2456–68.
19. Landgraf P, Rusu M, Sheridan R, et al. A mammalian microRNA expression atlas based on small RNA library sequencing. *Cell* 2007;**129**(7):1401–14.
20. Nelson PT, Baldwin DA, Kloosterman WP, et al. RAKE and LNA-ISH reveal microRNA expression and localization in archival human brain. *RNA* 2006;**12**(2):187–91.
21. Wilting SM, Steenbergen RD, Tijssen M, et al. Chromosomal signatures of a subset of high-grade premalignant cervical lesions closely resemble invasive carcinomas. *Cancer Res* 2009;**69**(2):647–55.
22. Agirre X, Vilas-Zornoza A, Jimenez-Velasco A, et al. Epigenetic silencing of the tumor suppressor microRNA Hsa-miR-124a regulates CDK6 expression and confers a poor prognosis in acute lymphoblastic leukemia. *Cancer Res* 2009;**69**(10):4443–53.
23. Furuta M, Kozaki KI, Tanaka S, Arii S, Imoto I, Inazawa J. MiR-124 and miR-203 are epigenetically silenced tumor-suppressive microRNAs in hepatocellular carcinoma. *Carcinogenesis* 2010;**31**(5):766–76.
24. Karginov FV, Conaco C, Xuan Z, et al. A biochemical approach to identifying microRNA targets. *Proc Natl Acad Sci USA* 2007;**104**(49):19291–6.
25. Dong P, Nabeshima K, Nishimura N, et al. Overexpression and diffuse expression pattern of IQGAP1 at invasion fronts are independent prognostic parameters in ovarian carcinomas. *Cancer Lett* 2006;**243**(1):120–7.
26. Mataraza JM, Briggs MW, Li Z, et al. IQGAP1 promotes cell motility and invasion. *J Biol Chem* 2003;**278**(42): 41237–45.
27. Takemoto H, Doki Y, Shiozaki H, et al. Localization of IQGAP1 is inversely correlated with intercellular adhesion mediated by e-cadherin in gastric cancers. *Int J Cancer* 2001;**91**(6): 783–8.
28. Jadeski L, Mataraza JM, Jeong HW, Li Z, Sacks DB. IQGAP1 stimulates proliferation and enhances tumorigenesis of human breast epithelial cells. *J Biol Chem* 2008;**283**(2): 1008–17.
29. Lee MR, Kim JS, Kim KS. MiR-124a is important for migratory cell fate transition during gastrulation of human embryonic stem cells. *Stem cells* 2010;**28**(9):1550–9.
30. Kawataki T, Yamane T, Naganuma H, et al. Laminin isoforms and their integrin receptors in glioma cell migration and invasiveness: evidence for a role of alpha5-laminin(s) and alpha3beta1 integrin. *Exp Cell Res* 2007;**313**(18): 3819–31.
31. Piao Y, Lu L, de Groot J. AMPA receptors promote perivascular glioma invasion via beta1 integrin-dependent adhesion to the extracellular matrix. *Neuro Oncol* 2009;**11**(3):260–73.
32. Staquicini FI, Dias-Neto E, Li J, et al. Discovery of a functional protein complex of netrin-4, laminin gamma1 chain, and integrin alpha6beta1 in mouse neural stem cells. *Proc Natl Acad Sci USA* 2009;**106**(8):2903–8.
33. Hendrickson DG, Hogan DJ, Herschlag D, Ferrell JE, Brown PO. Systematic identification of mRNAs recruited to argonaute 2 by specific microRNAs and corresponding changes in transcript abundance. *PLoS One* 2008;**3**(5):e2126.
34. Hendrickson DG, Hogan DJ, McCullough HL, et al. Concordant regulation of translation and mRNA abundance for hundreds of targets of a human microRNA. *PLoS Biol* 2009;**7**(11):e1000238.
35. Komatani H, Sugita Y, Arakawa F, Ohshima K, Shigemori M. Expression of CXCL12 on pseudopalisading cells and proliferating microvessels in glioblastomas: an accelerated growth factor in glioblastomas. *Int J Oncol* 2009;**34**(3): 665–72.
36. Brat DJ, Castellano-Sanchez AA, Hunter SB, et al. Pseudopalisades in glioblastoma are hypoxic, express extracellular matrix proteases, and are formed by an actively migrating cell population. *Cancer Res* 2004;**64**(3):920–7.
37. Lujambio A, Esteller M. CpG island hypermethylation of tumor suppressor microRNAs in human cancer. *Cell Cycle* 2007;**6**(12):1455–9.
38. Lujambio A, Ropero S, Ballestar E, et al. Genetic unmasking of an epigenetically silenced microRNA in human cancer cells. *Cancer Res* 2007;**67**(4):1424–9.

39. Fuller GN, Su X, Price RE, et al. Many human medulloblastoma tumors overexpress repressor element-1 silencing transcription (REST)/neuron-restrictive silencer factor, which can be functionally countered by REST-VP16. *Mol Cancer Ther* 2005;**4**(3):343–9.

40. Visvanathan J, Lee S, Lee B, Lee JW, Lee SK. The microRNA miR-124 antagonizes the anti-neural REST/SCP1 pathway during embryonic CNS development. *Genes Dev* 2007;**21**(7):744–9.