miR-155 is up-regulated in primary and secondary glioblastoma and promotes tumour growth by inhibiting GABA receptors

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Abstract. An altered expression of microRNAs (miRNAs) contributes both to the development of cancer and to the progression of the disease. Malignant tumours and tumour cell lines have widespread deregulated expressions of miRNAs compared to normal tissues. In this study, we investigated the expression profiles of 340 mammalian miRNAs in 93 cases of multiform glioblastoma (primary and secondary glioblastoma tumours), by means of DNA microarrays. We show that the expression profiles of 10 miRNAs can distinguish primary from secondary glioblastoma types. Moreover, we found elevated miR-155 levels in primary and secondary glioblastoma tissues as well as in glioblastoma primary cultures. We hypothesised that γ-aminobutyric acid A receptor 1 (GABRA1) is a miR-155 target, and studied the correlation between miR-155 up-regulation and the GABRA1 protein in cultured glioblastoma cells by miRNA silencing. We show that a decrease in miR-155 expression to normal levels restores the expression of GABRA1, making glioblastoma cells sensitive to signals that inhibit cell proliferation mediated by GABRA1. In conclusion, the expression patterns of different miRNAs characterise primary and secondary glioblastomas. The aberrant overexpression of miR-155 contributes to the malignant phenotype of glioblastoma cells removing growth inhibition.

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Introduction

microRNAs (miRNAs) are small (21-25 nucleotides in length) non-coding RNA molecules that negatively regulate protein expression by targeting the mRNA of protein-coding genes for either cleavage or repression of translation. They are involved in fundamental cellular processes such as brain development and neuronal differentiation (1-3). During brain development and neuronal differentiation, miRNAs are transcriptionally regulated and show a temporal wave of expression (4). By studying miRNA expression profiles in several cancer types and their activity on tumour suppressor genes or oncogenes, it was found that miRNAs are linked to cancer development (5-11). miR-155 is one of the miRNAs most frequently implicated in cancers. Transgenic mouse studies have demonstrated that B-cell-targeted expression of miR-155 leads to the development of B-cell malignancies (12). A number of miRNA profiling studies have shown elevated levels of miR-155 in a wide array of cancers, including lymphomas (12-16). Furthermore, it has been shown that a high expression of miR-155 in lung cancer correlates with poor survival rates (17). In glioblastoma multiforme (GBM) an increased expression of miR-21 with anti-apoptotic functions has been found (18). Huse *et al* reported the identification of miR-26a as a direct regulator of phosphatase and tensin homologue (PTEN) expression in high-grade glioma (19).

Glioblastomas represent 12-15% of intracranial tumours and 60-75% of astrocytic tumours. According to the current WHO classification of brain tumours glioblstoma is a grade IV astrocytoma. Glioblastomas can develop along two distinct clinical pathways: primary glioblastomas with no pathological precursor lesion, which have clinical prodromes of <3 months and secondary glioblastomas which may develop through progression from diffuse astrocytoma (WHO grade II) or anaplastic astrocytoma (WHO grade III). Secondary glioblastomas are less frequent than primary glioblastomas and typically develop in younger patients (mean age, 45 years). The time taken to progress from diffuse astrocytoma to GBM varies

considerably, with intervals ranging from <1 year to >10 years (mean interval, 4-5 years) (20). After the molecular analysis of glioblastoma tissues, the amplification of the epidermal growth factor receptor (EGFR) has been identified as a genetic marker for primary glioblastoma. TP53 has also been identified as a marker for low-grade astrocytoma and secondary glioblastoma. Different transcriptional profiles reflect biological differences between primary and secondary glioblastomas (21). At the same time, the strikingly similar phenotype of glioblastomas is reflected in common genetic lesions, such as loss of PTEN and cyclin-dependent kinase (CDK) inhibitor 2A (CDKN2A), as well as amplification/overexpression of CDK4 and murine double minute 2 (MDM2) (22).

The purpose of this study was to find a 'molecular signature' in order to distinguish primary from secondary glioblastomas. For this reason, we evaluated the expression of 340 miRNAs and investigated the crucial role of miR155 in gliomagenesis using 5 high-grade gliomas in primary culture.

Materials and methods

Human tissue samples. Ninety-three patients with glioblastoma tumours were admitted to our institutions; 66 patients had primary glioblastoma while the remaining 27 had secondary glioblastoma.

Fresh-frozen human glioblastoma samples were obtained for further analysis during surgical procedures and subjected to RNA extraction soon after arrival in the laboratory. All human materials were used in accordance with the policies of local institutional review boards. With written informed consent obtained from all patients, tumour RNA was obtained for molecular analysis. Patients ranged in age from 19-75 years with a mean age of 61 years, with >80% of patients being older than 50 years.

Tumours were classified according to the last WHO classification of central nervous tumours (20), and clinical histories recorded. Tumours were diagnosed as primary glioblastoma if at the time of the first surgery a prior history of glioma was not documented, and as secondary glioblastoma if a histological diagnosis of low-grade astrocytoma preceded by at least 1 year the diagnosis of glioblastoma.

Due to the known genotypic differences between oligodendroglial and astrocytic tumours, lesions with histological evidence of a prominent oligodendroglial component were excluded. Moreover, the 'giant cell' variant of glioblastoma was excluded as it has a genetic pattern overlapping that of primary and secondary glioblastoma (23).

For each case, the data including gender, age, location of tumour and survival time were collected and deposited at the Gene Expression Omnibus (GEO) database at the National Centre for Biotechnology Information (NCBI). A data summary is reported in Table I.

Microarray fabrication. Standard microscope glass slides from Sigma were activated with glycidyloxipropyltrimethoxysilane (GOPTS) as previously described (24). These activated epoxy glass slides immobilise amino-modified oligonucleotide DNA.

A 340 custom oligo array, comprising positive and negative control probes, was built where each DNA probe was complementary to a corresponding full length of mature miRNA. Detailed information on microarray protocols can be found at the GEO database at the NCBI.

miRNA extraction labelling and hybridisation. Total RNA was extracted from tissue using TRIzol™ (Invitrogen). After pulverizing the tissue in a stainless steel mortar and pestle chilled with liquid nitrogen, miRNA was isolated using a PureLink™ miRNA Isolation kit (Invitrogen) following the manufacturer's instructions.

The NCode™ miRNA Labeling System (Invitrogen) was used according to manufacturer's instructions and miRNAs were tagged, hybridised and then placed onto the microarray. After washing, the array was hybridised with Alexa Fluor® 3 and AlexaFluor® 5 capture reagents. Following further washing, the array was scanned using an Affimetrix 428 array scanner.

Real-time quantitative PCR (RTi-qPCR) and Northern blot analysis. Total RNA was reverse-transcribed to cDNA using primers gene-specific to all of the miRNAs (25). The expression of the miRNA precursors was determined using RTi-qPCR as previously described (26) with several modifications. The master mix contained 0.5 ml of 10X PCR buffer, 0.7 ml of 25 mM $MgCl_2$, 0.1 ml of 12.5 mM dNTPs, 0.01 ml UNG, 0.025 ml Amplitaq Gold DNA polymerase, 0.5 ml of diluted cDNA (1:50) and water.

All the PCR reagents were from the SYBR-Green core reagent kit (Applied Biosystems). The expression of each miRNA relative to U6 RNA was determined using the $2^{\Delta\Delta CT}$ method. Northern blot analyses were carried out with total RNA as previously described (25).

Culture of primary glioblastoma cells and siRNA. Early-passage (passage 3) cultures from 5 independent human high-grade gliomas were obtained by fresh surgical samples (3 primary GBM and 2 secondary GBM). Three cultures were established from each of the 5 high-grade gliomas, to produce a total of 15 early-passage cultures (passage 3). Tumour specimens were minced and suspended in 10 ml of Gey's balanced salt solution in a 50-ml tube. After centrifugation at 50 x g for 5 min, the supernatant was removed and 10 ml of 0.003% trypsin (Gibco, Berkeley, CA) in a balanced salt solution was added. Following incubation at 37˚C for 10 min, the upper 2 ml of the supernatant was discarded and the remaining contents were transferred to a 50 ml flask. An additional 10 ml of 0.003% trypsin was added and the solution was agitated with a magnetic stirrer for 10 min at 37˚C. After settling for 1 min, the supernatant was removed and refrigerated. The remaining portion was subjected twice to the same process. The supernatant collected from the 3 trypsinization procedures was filtered through a steel mesh filter (no. 160). The filtered single-cell suspension was centrifuged at 50 x g for 5 min and the supernatant was discarded. The cell button was suspended in 5-10 ml of medium. Following a cell count, appropriate dilutions were used to seed culture flasks (27). Cells were transfected according to the method described by Tönges *et al*, using stearyl-R8 like trasfection reagent (28) (stearyl-R8 was chemically synthesised). The results showed that this novel transfection method yielded performances comparable to cationic liposome-mediated transfection for siRNA, while being less cytotoxic in primary neurons. The

Table I. A summary of clinical information regarding the patients enrolled in this study.

Clinical data	Values
Glioblastoma	93
primary glioblastoma	66
secondary glioblastoma	27
developed by:	
diffuse astrocytoma	12
anaplastic astrocytoma	15
Age (years, mean values)	58.7
primary glioblastoma	64.3
secondary glioblastoma	44.9
Male/female ratio	1.21
Tumour location	
sub cortical white matter of the cerebral hemispheres	93
primary glioblastoma sublocalization	
temporal	10
parietal	15
frontal	14
occipital	10
fronto-temporal	17
secondary glioblastoma sublocalization	
temporal	4
parietal	4
frontal	6
occipital	3
fronto-temporal	10
Survival (months, mean values)	
primary glioblastoma	4.9
secondary glioblastoma	7.8

reduced stearyl-octarginine concentration required for efficient transfection is the likely explanation for the low cytotoxic side-effects seen in the primary culture (29).

Cytotoxicity assay. The conversion of 3-(4.5-dimethylthiazol-2-yl)-2.5-diphenol tetrazolium bromide (MTT) by cells was used as an indicator of cell numbers as previously described (30). This method measured the reduction of MTT by active mitochondria, which resulted in a colour change measured at a 550-nm wavelength.

Experiments were performed to define the linear range of the assay. A positive correlation was observed up to 50,000 cells per well (data not shown). Increasing the concentration of heat-killed cells per well (killed by incubating at 70˚C for 15 min) caused no significant change in the absorbance. Therefore, this spectrophotometric method was deemed a valid technique for measuring the number of viable cells. All subsequent experiments performed were within the linear range of the assay. The percentage of cell survival was calculated as the absorbance ratio of treated to untreated cells.

Results

Gene expression profiling. The expression profiles of 340 mammalian miRNAs were analysed by DNA microarrays in 66 primary and 27 secondary glioblastomas and compared with normal adult brains. The latter was from commercially available normal brain tissue (FirstChoice® Total RNA, Applied Biosytems) and normal brain tissue obtained during surgery at our institutions. We selected all miRNAs expressed in at least 20 samples of the same group having a signal value >100, thus avoiding low-expression genes. Among them, miRNAs with a signal-to-noise ratio of >2.5 were selected for further analysis. Using these parameters, we obtained 86 selected gene features. Using Pearson's correlation co-efficients, average linkage clustering analysis was applied to all tissues, on the basis of the similarity of expression patterns over the 86 selected genes. As expected, this yielded 2 major clusters, one representing the primary glioblastoma group and the other the secondary glioblastoma group (Fig. 1a).

Subsequently, we independently filtered miRNAs showing differential expression between primary or secondary glioblastoma tumour tissue and normal brain tissue by means of ANOVA analysis. Parametric Student's t-test was applied, assuming equal variances, with a p-value cut-off of 0.01 and with multiple testing corrections (Benjamini-Hochberg false discovery rate). Using these restrictions we selected 12 gene features. miR-18b, miR-181c, miR-181a, miR-16, miR-15a and miR-128a where found to be down-regulated while miR-21, miR-155, miR-221, miR-347, miR-9 and miR-138 were found to be up-regulated in most glioblastoma samples. miR-21 and miR-155 were up-regulated in all samples analysed suggesting that these miRNAs are characteristic of glioblastoma.

The miRNAs with statistically significant differences were then grouped according to glioblastoma type (primary or secondary). The parametric Student's t-test was again applied assuming equal variances with a p-value cut-off of 0.01 and with multiple testing corrections (Benjamini-Hochberg false discovery rate). This restriction tested all previously selected genes and identified 10 gene features. Approximately 1.0% of the identified genes were expected to pass the restriction by chance. Using a standard leave-one-out cross-validation procedure, we found the 10 gene feature signatures to be significantly correlated with glioma subtype (p=0.01) (Fig. 1b). We determined whether machine-learning classifiers predict subtype in the profiles of glioma tissues. We tested multiple classification methods including classification and regression tree (CART), k-nearest neighbor (k-NN), weighted voting (WV) and support vector machine (SVM).

We were able to distinguish primary from secondary glioblastoma types based solely on these 10 miRNAs gene expression profiles as follows: high levels of miR-221 and low levels of miR-128a, miR-181a and miR-181c (primary glioblastomas); elevated levels of miR-9, miR-138, miR-347 and low levels of miR-15a and miR-16 (secondary glioblastomas, Fig. 1b). miR-21, miR-221, miR-155 were strongly elevated in the primary glioblastoma samples tested, as well as in the 5 early-passage cultures established from 5 patients.

RTi-qPCR. Up- or down-regulated miRNAs were validated by RTi-qPCR in 15 randomly chosen primary glioblastoma

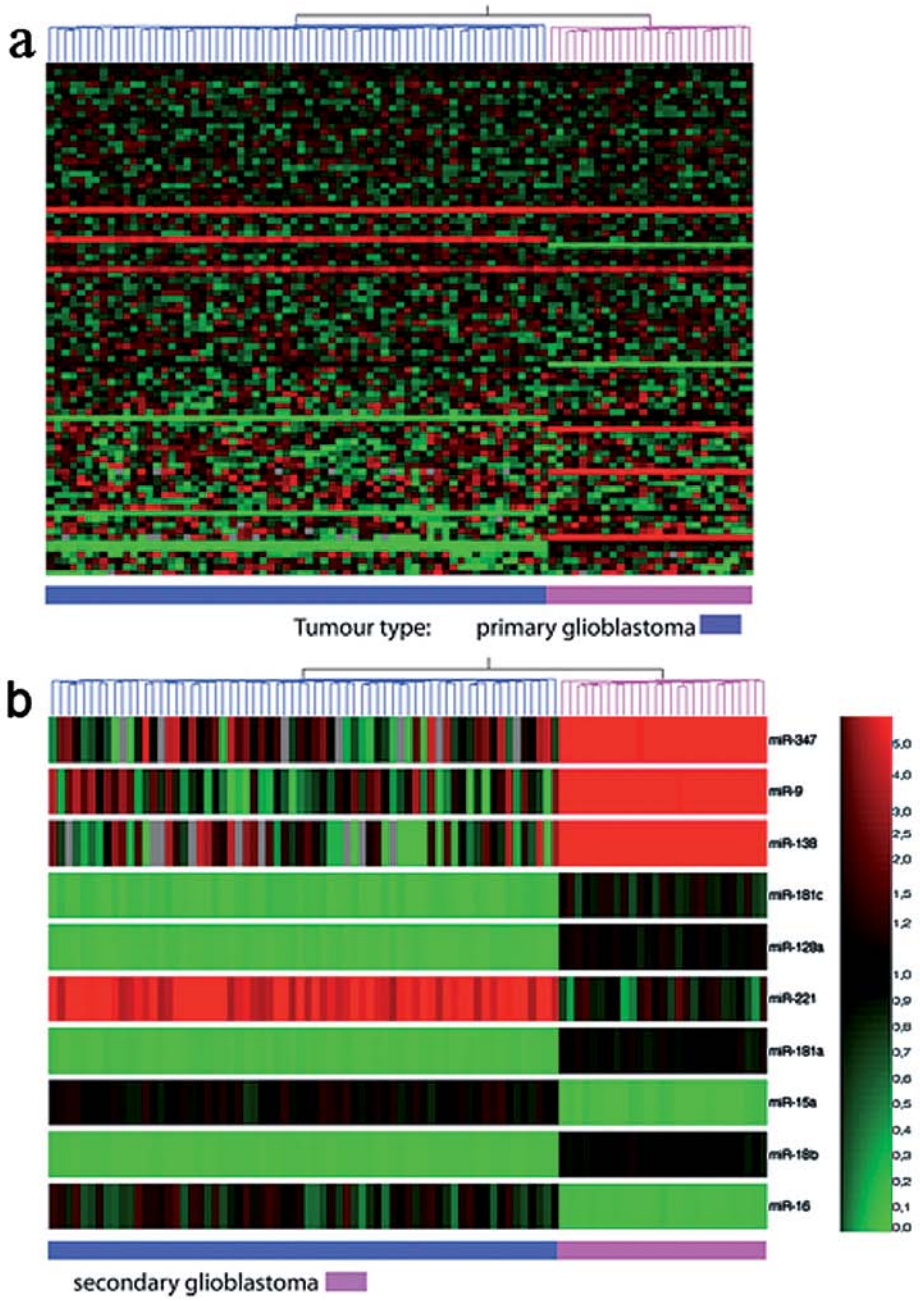

Figure 1. Cluster analysis. (a) The tree generated by a cluster analysis performed on 86 miRNAs showing the separation of primary glioblastoma from secondary glioblastoma tumours. (b) The tree generated by a cluster analysis performed on 10 miRNAs showing a clear separation of primary glioblastoma from secondary glioblastoma tumours.

tissues, and in 15 randomly chosen secondary glioblastoma tissues vs. normal adult brain tissue as the control. We selected a representative cluster of both up- and down-regulated genes in both primary and secondary glioblastoma. In some cases, we obtained a greater difference in respect to the microarray data (Fig. 2). The expression of each miRNA relative to U6 RNA was determined using the $2^{\Delta\Delta Ct}$ method.

Gene silencing. Previous studies have demonstrated that miR-21 is up-regulated in primary brain tumours and acts as an anti-apoptotic factor in glioblastoma cells (13). In the present study, we demonstrated that in addition to miR-21, miR-155 was also up-regulated in all the glioblastoma tumours analysed. We therefore focused our attention on miR-155 up-regulation.

The targets of miR-155 include the γ-aminobutyric acid (GABA) A receptor 1 (GABRA1), suppressor of cytokine signaling 1 (SOCS1), adenomatous polyposis coli (APC), and WEE1. We tried to verify whether miR-155 suppression affected its targets by means of RTi-qPCR in primary cultures. One day after miR-155 silencing, no changes were reported in SOCS1, APC and WEE1 expression while an increase in the GABRA1 mRNA level was observed. Labrakakis *et al* and Betel *et al* suggested a link between the expression of GABA receptors and the growth of glioma cells, since the loss of GABA receptors parallels the uninhibited growth typical of

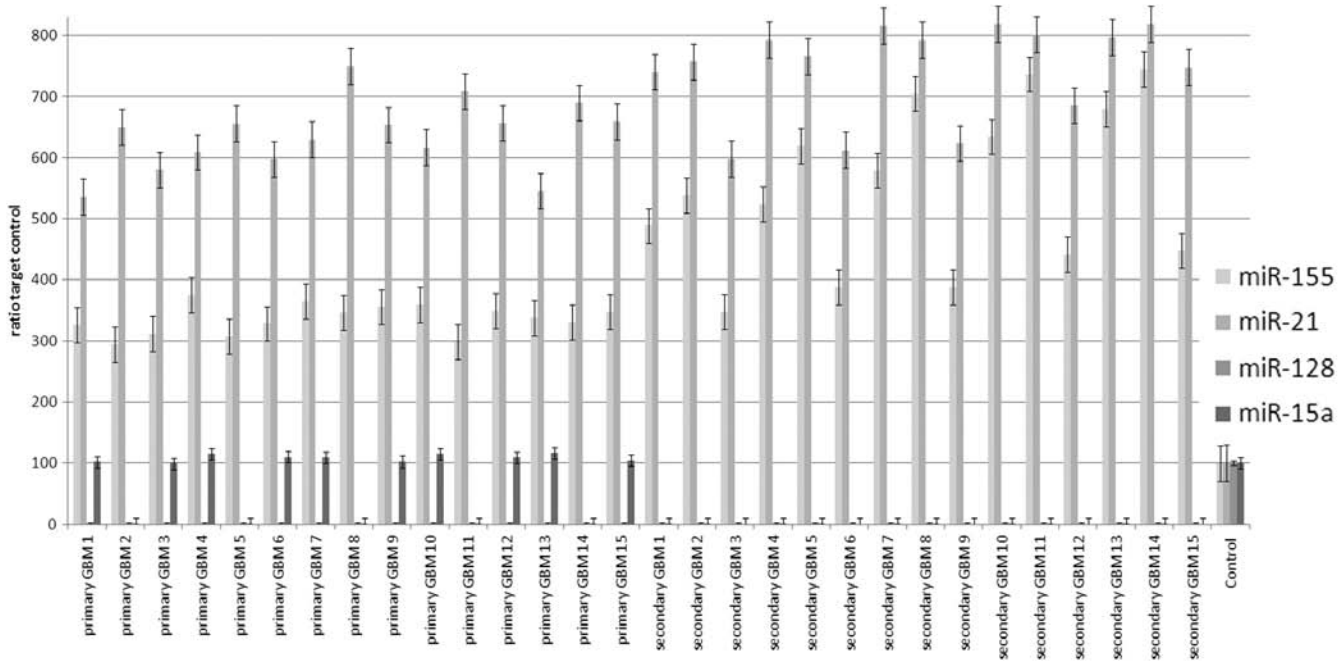

Figure 2. Real-time analysis. Microarray expression data was confirmed by real-time quantitative PCR on miR-155, miR-21, miR-128a and miR-15a. The Pfaffl method was adopted using the U6 gene as the reference gene. The ratio was calculated by the following formula: Ratio = $(E_{target})^{\Delta Ct\ target\ (control\text{-}test)}/(E_{reference})^{\Delta Ct\ reference\ (control\text{-}test)}$; ($p<0.01$). GBM, glioblastoma multiforme.

malignant tumours and immortalised cell lines (31,32). We investigated the correlation between GABRA1 down-regulation and miR-155 up-regulation and their implications on cell proliferation.

Human glioblastoma cells were GABRA1-negative since Northern blot analysis showed no expression of GABRA1 mRNA in glioblastoma primary cultures (Fig. 3a) and, accordingly, immunohistochemical results showed no expression of GABRA1 in paraffin-embedded glioblastoma tissue sections (data not shown). In order to study the biological significance of miR-155 up-regulation and its correlation with GABRA1 expression, we used a loss-of-function approach. A methodology using 2'-O-methyloligoribonucleotides as sequence-specific inhibitors of miRNA function and miRNA-directed RISC activity has previously been reported (33). These molecules stoichiometrically bind and irreversibly inactivate miRNAs, providing a valuable means of disrupting the function of a single miRNA *in vitro* and *in vivo*.

In order to test whether miR-155 suppression alters GABRA1 expression, we transfected the 2'-O-methyloligonucleotide complementary to miR-155 into primary cultures and performed Northern blot analysis one day after transfection. The results showed that the miRNA target became undetectable after the introduction of 2'-O-methyloligonucleotide in the low nanomolar range used and that miR-155 suppression resulted in an increase in GABRA1 mRNA expression (Fig. 3). This effect was sequence-specific. miR-155 was blocked by the corresponding antisense 2'-O-methyloligonucleotide (2'OMe-miR-155), but not by an unrelated 2'-O-methyloligonucleotide (2'OMe-EGFP) (Fig. 3b). These results were confirmed using Northern blot analysis for GABRA1 (Fig. 3c). Eight hours after transfection, miR-155-suppressed cells were stimulated with GABA and the cell number was evaluated by MTT assay and plate count. In cells transfected with 2'OMe-miR-155, GABA induced a significant decrease in cell number, whilst no response was observed in cells transfected with 2'OMe-EGFP (Fig. 4). These results strongly suggest that in glioblastoma cells, the up-regulation of miR-155 may be linked to the down-regulation of GABRA1 and to the loss of the anti-proliferative effects of GABA.

Discussion

We studied the expression profile of 340 miRNAs by means of a microarray methodology. Using a miRNA expression profiles, we were able to distinguish between primary and secondary glioblastomas, and the up-regulation of both miR-21 and miR-155 were clear indicators of the tumoural state.

Starting with expression data relating to all genes studied, we selected those having a significant difference between primary and secondary glioblastomas. When cluster analysis was performed using the expression of only these genes, we were able to divide glioblastoma tumours in two subclasses coinciding with neuroradiological and histological primary and secondary glioblastoma.

miR-21 was up-regulated in primary and secondary glioblastoma and in all the cell lines tested. We confirmed the array-identified up-regulation of miR-21 by RTi-qPCR, obtaining a greater difference with respect to the microarray data. miR-21 expression was increased from 10 to 100-fold in glioblastoma tissue compared with control non-neoplastic brain tissue.

Previous studies have shown that primary brain tumours, such as anaplastic astrocytoma, oligoastrocytoma, oligo-

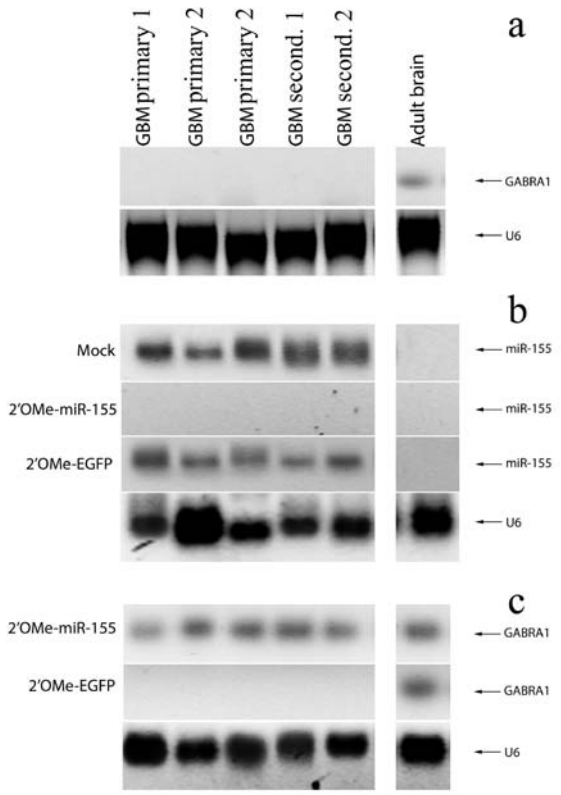

Figure 3. Northern blot analysis. (a) Northern blot analysis showing GABRA1 expression. The same blot was re-probed with U6 as the loading control. (b) Northern blot analysis showing the expression of miR-155 after transfection with the vehicle control (mock), with a 2'-O-methyloligonucleotide complementary to miR-155 (2'OMe-miR-155) and an irrelevant 2'-O-methyloligonucleotide (2'OMe-EGFP). The same blot was re-probed with U6 as the loading control. (c) Northern blot analysis showing the expression of GABRA1 after trasfection with a 2'-O-methyloligonucleotide complementary to miR-155 (2'OMe-miR-155) and an irrelevant 2'-O-methyloligonucleotide (2'OMe-EGFP). The same blot was re-probed with U6 as the loading control (each blot was re-probed with the U6 probe but only one blot of U6 for each treatment is shown). GBM, glioblastoma multiforme; GABRA1, γ-aminobutyric acid A receptor.

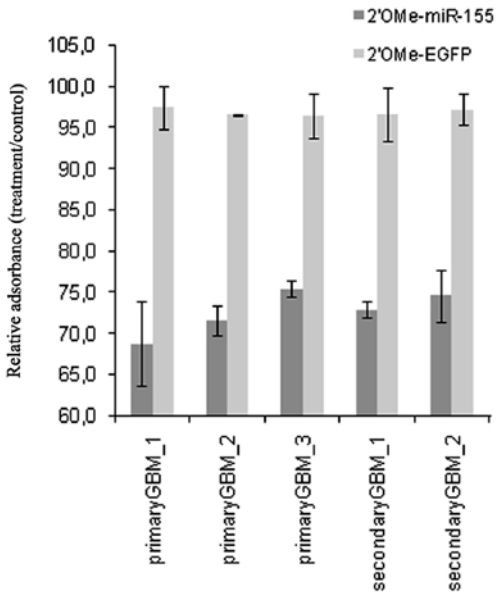

Figure 4. The sensitivity of the cells to γ-aminobutyric acid (GABA) stimulation. Cells were transfected with a 2'-O-methyloligonucleotide complementary to miR-155 (2'OMe-miR-155) and an irrelevant 2'-O-methyloligonucleotide (2'OMe-EGFP). The data represent the means ± SD from 4 different experiments with 8 replicate wells in each microtitre plate ($p<0.01$). GBM, glioblastoma multiforme.

dendroglioma and medulloblastoma are characterised by an increase in miR-21 expression; however, very high levels of this miRNA are more typical of glioblastomas (18,34-38).

Moreover, it has been demonstrated that miR-21 acts as an anti-apoptotic factor in glioblastoma cells, suggesting that the aberrantly increased expression of miR-21 may down-regulate the translation or stability of mRNA(s) coding for apoptosis-related genes, although the precise targets of miR-21 remains elusive (18).

It can be postulated that the aberrant increased expression of this miRNA may block the expression of gene products that promote normal glial differentiation or that induce apoptosis, holding tumour cells in an inappropriately primitive and proliferative developmental state.

miR-155 potential targets include the tumour suppressor genes, SOCS1 and APC, and the kinase WEE1, which blocks the activity of Cdc2 and prevents entry into mitosis. The hypoxia-inducible factor (HIF)1A is also a predicted target. Interestingly, among predicted genes, the tripartite motif-containing protein 2 (TRIM2), the proto-oncogene, sphingosine kinase 1 (SKI), and the RAS homologues, RAB6A and RAB6C, were found as potential targets of both miR-21 and miR-155 (32).

One of the miR-155 targets is GABRA (31). Labrakakis *et al* suggested a link between the expression of GABA receptors and the growth of glioma cells since the disappearance of functional GABA receptor parallels the uninhibited growth typical of malignant tumours and immortal cell lines (32).

We studied the correlation between GABRA1 down-regulation and miR-155 up-regulation, and the implications for cell proliferation control. Our data suggest that in glioblastoma cells one of the effects of miR-155 up-regulation may be the down-regulation of GABRA1, which renders tumour cells unresponsive to GABA cell proliferation inhibition, playing an important role in the chain of events that induce uninhibited cellular growth in malignant tumours.

In conclusion, although a different pattern of miRNA expression characterises primary and secondary glioblastoma, these tumours have the common up-regulation of both miR-21 and miR-155, which hold tumour cells in an inappropriately primitive and proliferative developmental state.

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