MOLECULAR ONCOLOGY 9 (2015) 704–714

available at www.sciencedirect.com

ScienceDirect

www.elsevier.com/locate/molonc

Prediction of clinical outcome in glioblastoma using a biologically relevant nine-microRNA signature

Josie Hayes[a], *Helene Thygesen*[a], *Charlotte Tumilson*[b], *Alastair Droop*[a], *Marjorie Boissinot*[a], *Thomas A. Hughes*[c], *David Westhead*[d], *Jane E. Alder*[b], *Lisa Shaw*[b], *Susan C. Short*[a], *Sean E. Lawler*[e,*]

[a]*Leeds Institute of Cancer and Pathology, St James's University Hospital, Leeds LS9 7TF, UK*
[b]*School of Pharmacy and Biomedical Sciences, University of Central Lancashire, Preston, Lancashire PR1 2HE, UK*
[c]*Leeds Institute of Biomedical and Clinical Sciences, St James's University Hospital, Leeds LS9 7TF, UK*
[d]*School of Molecular and Cellular Biology, Faculty of Biological Sciences, University of Leeds, Leeds LS2 9JT, UK*
[e]*Harvey Cushing Neurooncology Laboratories, Department of Neurosurgery, Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02115, USA*

ARTICLE INFO

Article history:
Received 18 August 2014
Received in revised form
29 October 2014
Accepted 14 November 2014
Available online 28 November 2014

Keywords:
Glioblastoma
Prognosis
MicroRNA
Signature
TCGA

ABSTRACT

Background: Glioblastoma is the most aggressive primary brain tumor, and is associated with a very poor prognosis. In this study we investigated the potential of microRNA expression profiles to predict survival in this challenging disease.
Methods: MicroRNA and mRNA expression data from glioblastoma ($n = 475$) and grade II and III glioma ($n = 178$) were accessed from The Cancer Genome Atlas. LASSO regression models were used to identify a prognostic microRNA signature. Functionally relevant targets of microRNAs were determined using microRNA target prediction, experimental validation and correlation of microRNA and mRNA expression data.
Results: A 9-microRNA prognostic signature was identified which stratified patients into risk groups strongly associated with survival ($p = 2.26e{-}09$), significant in all glioblastoma subtypes except the non-G-CIMP proneural group. The statistical significance of the microRNA signature was higher than *MGMT* methylation in temozolomide treated tumors. The 9-microRNA risk score was validated in an independent dataset ($p = 4.50e{-}02$) and also stratified patients into high- and low-risk groups in lower grade glioma ($p = 5.20e{-}03$). The majority of the 9 microRNAs have been previously linked to glioblastoma biology or treatment response. Integration of the expression patterns of predicted microRNA targets revealed a number of relevant microRNA/target pairs, which were validated in cell lines.
Conclusions: We have identified a novel, biologically relevant microRNA signature that stratifies high- and low-risk patients in glioblastoma. MicroRNA/mRNA interactions identified within the signature point to novel regulatory networks. This is the first study to formulate a survival risk score for glioblastoma which consists of microRNAs

* *Corresponding author*. Tel.: +1 617 525 5066; fax: +1 617 525 5067.
E-mail address: slawler@partners.org (S.E. Lawler).
http://dx.doi.org/10.1016/j.molonc.2014.11.004

associated with glioblastoma biology and/or treatment response, indicating a functionally relevant signature.

1. Introduction

Glioblastoma is a primary central nervous system tumor with a particularly poor outcome (Louis et al., 2007; Stupp and Roila, 2009). Standard treatment involves surgery followed by radiotherapy and chemotherapy with temozolomide (Louis et al., 2007; Stupp and Roila, 2009). Current molecular prognostic markers include *IDH1/2* (isocitrate dehydrogenase 1/2) mutation and *MGMT* (O6-methylguanine-DNA methyltransferase) promoter methylation, which confer improved prognosis and relative sensitivity to temozolomide treatment respectively (Riemenschneider et al., 2010). Additional prognostic indicators are age and Karnofsky performance score (KPS) (Chaichana et al., 2013). Glioblastoma primarily occurs *de novo* with no evidence of progression from a lower grade tumor. However, approximately 5%, known as secondary glioblastoma, arise by progression from a lower grade astrocytoma (Ohgaki and Kleihues, 2007). Secondary glioblastoma is often associated with mutations in *IDH1/2* (Parsons et al., 2008).

MicroRNAs are 22–24 nucleotide non-coding RNAs, which downregulate translation by targeting messenger RNAs (mRNAs) (Krol et al., 2010). MicroRNA expression signatures can define tumor types and molecular subgroups, and are prognostic in some cancers (Calin and Croce, 2006; Hayes et al., 2014; Kim et al., 2011; Volinia et al., 2006). Molecular profiling studies have shown differential microRNA expression in glioblastoma compared to normal brain tissue, and also between glioblastoma subtypes (Kim et al., 2011; Lang et al., 2012). Several individual microRNAs have been associated with glioblastoma prognosis (Mizoguchi et al., 2012), but it is likely that multiple microRNAs will provide a more statistically robust approach. Previous prognostic signatures for GBM have been designed {Lakomy:2011ju}{Srinivasan:2011fh}{Zhang:2012iq}, although the microRNAs employed are not consistent between studies.

A novel methodology, known as LASSO (least absolute shrinkage and selection operator (Tibshirani, 1996)), was used, with glioblastoma data from The Cancer Genome Atlas (TCGA) ("The Cancer Genome Atlas – Data Portal, tcga-data.nci.nih.gov"), to identify a 9-microRNA prognostic signature. The 9 microRNAs were then used to generate a risk score algorithm suitable for clinical prognostic stratification. The signature separated patients according to outcome, was relevant in temozolomide treatment and was validated in an independent dataset. Although other microRNA prognostic signatures have been identified in glioblastoma, this is the first to use the whole TCGA dataset; it is relevant across subtypes and in treatment, and is the first to be validated in an independent dataset. Moreover, the signature microRNAs have been previously implicated in glioblastoma, with known functional roles, further supporting the relevance of the signature. Thus we have identified a functionally relevant, microRNA-based prognostic signature in glioblastoma.

2. Materials and methods

2.1. TCGA clinical information and expression data

Level 2 Agilent microRNA 8 × 15k microarray and G4520A microarray gene expression data plus clinical information for 475 glioblastoma and 10 unmatched non-tumor samples were downloaded from TCGA ("The Cancer Genome Atlas – Data Portal, tcga-data.nci.nih.gov") (accessed October 2012). Only patients treated with radiotherapy and some form of chemotherapy were selected (Table 1). Illumina HiSeq sequencing data (level 3, reads per million of total reads mapping to a mature microRNA) for microRNAs were downloaded for all samples with grade II or III glioma from TCGA ($n = 178$; 55 astrocytoma, 47 oligodendrocytoma, 75 oligodendroglioma, 1 not stated; 95 grade II, 112 grade III, 1 not stated).

Table 1 – Characteristics of patients used in the generation of the signature. The characteristics of the 475 patients included in the generation and testing of the model. There are more males in the study (62%), which is expected for a glioblastoma cohort. KPS was calculated prior to surgery. There were 26 IDH mutations recorded in this cohort although 117 did not have IDH mutation information.

Characteristic	Number of patients ($n = 475$)
Age (median = 59)	
<60 years	248
≥60 years	227
Gender	
Male	293
Female	182
Karnovsky performance score	
≤70	141
>70	220
Not available	114
Days to death/last follow-up (median 430 days)	
<450 days	301
≥450 days	174
≤30 days	20
Therapy	
TMZ	3
TMZ and radiation	187
Other	285

2.2. Statistical analysis of microRNA expression data in glioblastoma

Glioblastoma samples were assessed using a LASSO penalized regression analysis to predict survival using microRNA expression (Tibshirani, 1996) with leave-one-out cross-validation using R software (v2.15.1) and the Penalized package (Goeman, 2012.). A risk score was generated using the sum of microRNA expression values weighted by the coefficients from the LASSO regression, as described (Alencar et al., 2011).

This was: E_miR-n = expression of microRNA *n*.

Risk score = −0.044E_miR-370 + 0.062E_miR-124a + −0.066E_miR-145 + 0.005E_miR-34a + 0.015E_miR-10b + 0.092E_miR-148a + 0.162E_miR-222 + −0.032E_miR-9 + −0.021 E_miR-182.

The risk score was applied to all glioblastoma samples in the dataset and the samples separated into low- and high-risk groups using the median as a cut-off. A Cox regression model incorporating age and the log-rank test were used to assess overall survival (OS) of the two groups in the whole dataset, the molecular subtypes of glioblastoma (using published classification information (Brennan et al., 2013)) and temozolomide treated patients. The score was also assessed in progression-free survival (PFS). A statistical significance threshold of $p = 0.05$ was used throughout. Pearson's correlation coefficient was calculated for correlation of age with risk score. Multivariable Cox regression models for the risk groups and each of the following factors (separately); *MGMT* methylation, gender, IDH mutation, subtypes, extent of resection and KPS (at diagnosis) were used to compare the two predictors using TCGA data (Brennan et al., 2013).

2.3. Prognostic validation of the signature in an independent dataset

Frozen glioblastoma tissue was obtained from the Brain Tumour North West tissue bank, Royal Preston Hospital, UK. Total RNA was extracted using TRIZOL (Life Technologies, UK) according to the manufacturer's guidelines. 1 μg of total RNA was reverse transcribed using the NCode miRNA First-strand cDNA synthesis Kit (Life Technologies). Real-time PCR was performed using GoTaq qPCR Master Mix (Promega, UK) on an Applied Biosystems 7500 PCR Machine with U6 snRNA endogenous control. Average C_t values were calculated for each miRNA, then normalized to U6 average C_t values (ΔC_t). These ΔC_t values were used in the signature algorithm to create risk scores for each patient. One-tailed Cox regression was performed using these scores. The patients were separated according to the 60th percentile and the high- and low-risk groups assessed for association with survival using a one-tailed log-rank test.

2.4. Assessment of the 9-microRNA signature in lower grade glioma

MicroRNA expression for WHO Grade II and Grade III astrocytoma was based on sequencing reads per million mapping to a mature microRNA. Risk scores were calculated and significance assessed as above. The median of the lower grade dataset was recalculated and used to separate the samples into two groups.

2.5. Cell culture, transfection and validation of candidate microRNA targets

LN229 glioblastoma cells (ATCC) were cultured in DMEM containing 10% fetal bovine serum at 37 °C in 5% CO_2. Cells were transfected with 100 nM miR-9 mimic or scrambled control oligonucleotides (ThermoScientific, Waltham, USA), using 10 μl of Lipofectamine RNAiMAX (Life Technologies, Carlsbad, CA) per 2.5 ml of transfection mix in six-well plates containing 150,000 cells/well. RNA was extracted 48 h post-transfection (miRNeasy, Qiagen, Gaithersburg, MD) and first-strand synthesis done using SuperScript® II Reverse Transcriptase (Life Technologies). Quantitative PCR (qPCR) analyses were performed in triplicate with Taqman assays (Life Technologies).

2.6. Identifying predicted microRNA targets associated with survival

Gene expression was compared between two groups of patients from the extremes of survival in the TCGA dataset; poor prognosis (survival time < 115 days, $n = 14$, minimum KPS at diagnosis = 80) and good prognosis groups (survival time > 1825 days, $n = 14$). The LIMMA (linear models for microarray data) package was used to perform differential expression analysis, and the genes with a *p*-value of less than 0.05 and greater than 1.5-fold change in expression were used as input to RmiR version 1.14, an R-based program for assessment of microRNA targets (Favero v2.14). Gene ontology analysis was performed using Metacore v6.16 (Thomson Reuters) modified exact Fisher's test and pathways determined using DIANA miRpath (Vlachos et al., 2012) (one-tailed Fisher's exact test for enrichment of predicted microRNA targets). RmiR v1.14 was used to identify targets of the 9 microRNAs amongst the genes which were present in all databases of; Miranda (Miranda et al., 2006), Pictar (Krek et al., 2005) and Targetscan (Lewis et al., 2005) (as loaded by RmiR vignette). Correlation of microRNA and gene expression was performed using Spearman's correlation on all 475 glioblastoma samples.

3. Results

3.1. Identification of a 9-microRNA signature associated with prognosis in glioblastoma

In order to identify microRNAs associated with OS in glioblastoma, LASSO regression (Tibshirani, 1996) was performed using microRNA expression data (534 microRNAs, 1510 probes) for 475 glioblastomas. This method is optimized to hi-dimensional data (where there are more potential predictors than samples) allowing valid inclusion of the 9 microRNAs in the model. The method performs a sub-selection of microRNAs involved in survival by shrinkage of the regression coefficient through imposing a penalty proportional to their size. This results in most potential predictors being shrunk to

zero leaving a relatively small number with a weight of non-zero. These microRNAs may not be the only potential predictors in the set, because, if two predictors exhibit co-linearity, LASSO will choose the one that has the strongest association with response (which is not necessarily the only causal one, especially if the difference between the two predictors' degree of association with response is not significant) and the other will be given zero weight.

Using the LASSO method, 12 microRNA probes were identified with non-zero regression coefficients. This included two probes for miR-182, which differed in length by one nucleotide. The longer probe was used for the remainder of the study. Also a probe for miR-565 was identified that has since been excluded from miRBase (Griffiths-Jones et al., 2008) as it is classified as a tRNA fragment; this was not studied further. The LASSO model was refitted without these two probes resulting in a 9-microRNA signature (Table 2). MicroRNAs given a negative LASSO coefficient are positive predictors of survival and *vice versa*. Seven of the microRNAs were significantly differentially expressed in non-tumor tissue compared to glioblastoma (Table 2).

3.2. *A risk score combining expression values of the 9 microRNAs predicts survival*

A risk score was created using the regression coefficients from the LASSO analysis (see Methods) to weight the expression value of each of the 9 microRNAs. The risk score was then separated on the median (1.48 quantile normalized probe expression) to create high and low risk groups. The median survival time of the low-risk group was 13.1 months and the median of the high-risk group was 9.5 months. Risk score was associated with survival using log-rank test (Figure 1, $p = 2.26e{-}09$). Median expression of each signature microRNA in both groups is shown in Supplementary Figure S1.

Pearson's correlation of age with risk score showed a significant direct correlation ($R = 0.248$, $p = 4.13e{-}08$). Multivariable Cox regression of the risk group and age showed the risk group to be an independent predictor of survival irrespective of age (Group HR = 1.61, 95% CI = 1.30–1.99, $p = 1.40e{-}5$; Age HR = 1.03, CI = 1.02–1.04, $p = 2.50e{-}3$). As males have poorer outcome in glioblastoma (Krex et al., 2007), the risk score was evaluated according to gender, and was found to be similar in the male and female groups (median 1.48 in each group).

Table 2 – MicroRNAs associated with survival using the LASSO regression test. Significant ($p < 0.05$) results are shown in bold. Nine microRNAs were reported as non-zero coefficients, five were negatively associated with survival and four were positively associated with survival. Seven were differentially expressed in unmatched non-tumor samples compared to glioblastoma samples.

Mirna	LASSO penalized coefficient for risk score (log 2)	Fold change in GBM compared to non-tumor
miR-124a	0.062	**0.032**
miR-10b	0.015	**10.005**
miR-222	0.162	**0.278**
miR-34a	0.005	**3.121**
miR-182	−0.021	**3.708**
miR-148a	0.092	**2.752**
miR-145	−0.066	**0.541**
miR-370	−0.044	1.274
miR-9	−0.032	0.863

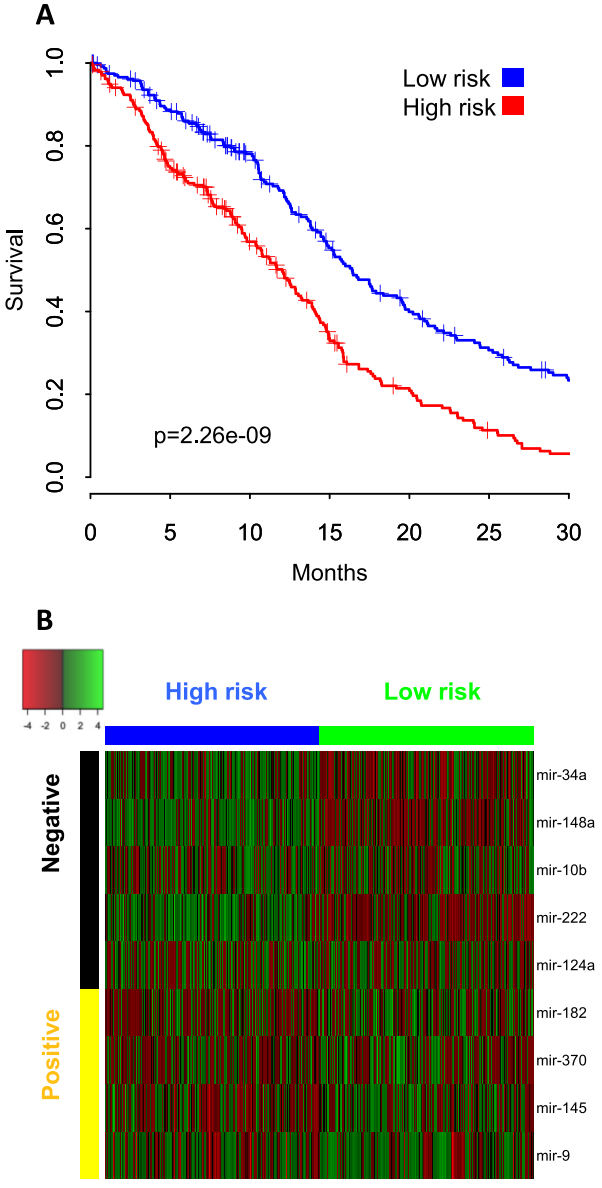

Figure 1 – The patient groups assigned to the high- and low-risk groups using the median as a threshold. A score for each patient was calculated using the microRNA expression signature and patients were separated into high and low risk groups using the median as a cut-off. A) The low-risk group has significantly longer survival times than those in the high-risk group by log-rank test. B) Expression patterns of the significant microRNAs in the high- and low-risk groups, as defined by the risk score, shown in a heatmap. The top five microRNAs in the heatmaps (black) act as more aggressive microRNAs, and the bottom four (yellow) are less aggressive microRNAs.

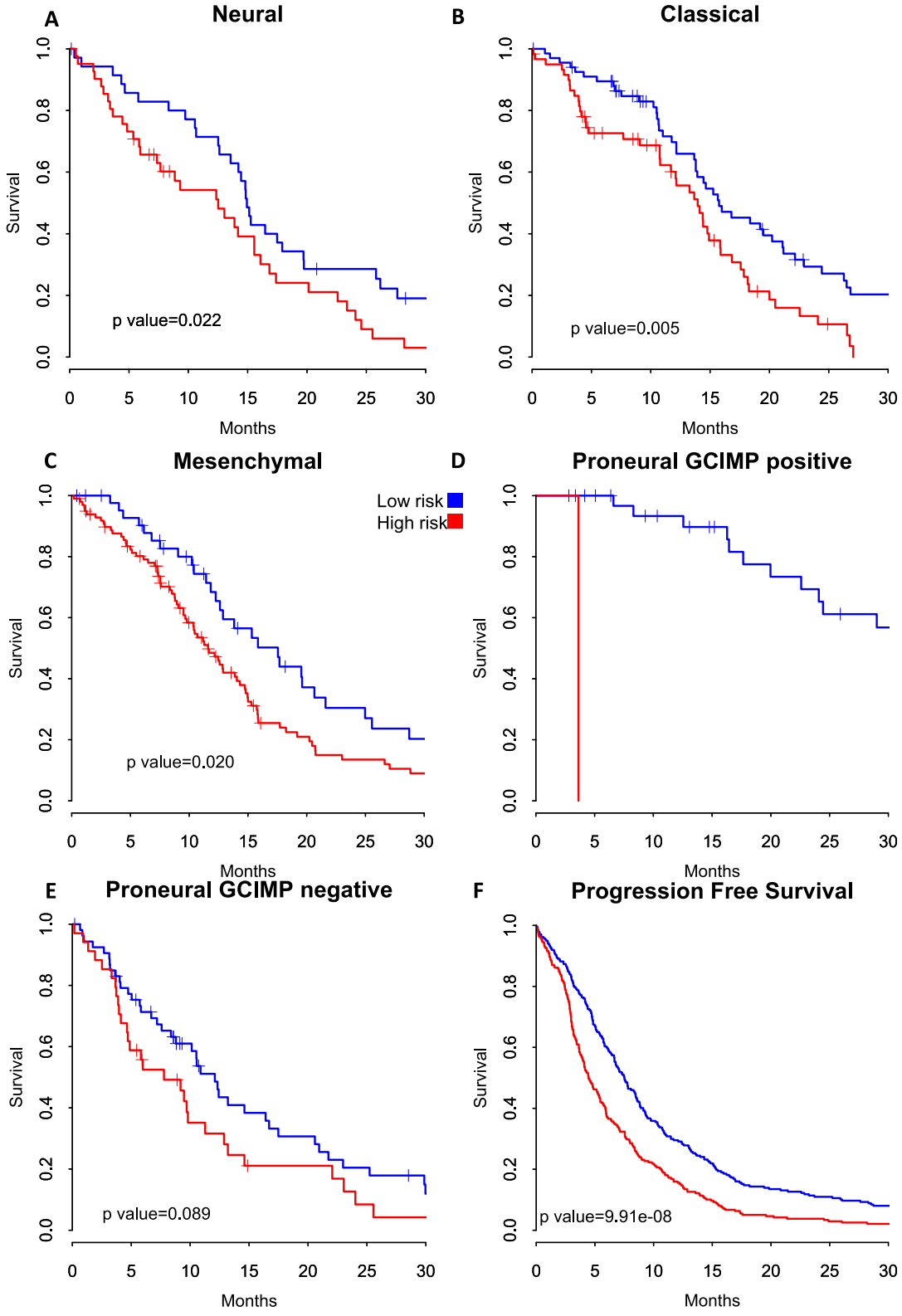

Figure 2 – Log-rank of the low-risk and high-risk groups in subgroups of glioblastoma. Risk scores were calculated with the same threshold as the whole cohort for each subtype of glioblastoma. The risk groups were significant by log-rank test (non-age adjusted) in all subtypes of glioblastoma but proneural G-CIMP negative (A–E). Risk score is also a significant predictor of progression free survival (F).

3.3. Assessment of the risk score in glioblastoma subtypes and in relation to other prognostic factors

We then determined the risk groups for each of the TCGA-defined glioblastoma molecular subtypes (Brennan et al., 2013): proneural G-CIMP positive ($n = 36$), proneural G-CIMP negative ($n = 88$), neural ($n = 77$), classical ($n = 128$) and mesenchymal ($n = 143$). Risk group was associated with survival in all subtypes except proneural G-CIMP negative (Figure 2A–E).

The groups were then fitted to a Cox regression model incorporating age in each patient subtype. The score remained significant in the classical (HR = 1.73, 95% CI = 1.13–2.64, $p = 0.011$) and neural (HR = 2.03, 95% CI = 1.23–3.38, $p = 0.007$) groups and age was a confounding factor in the mesenchymal group (HR = 1.46, 95% CI = 0.95–2.23, $p = 0.084$). The proneural G-CIMP positive group could not be calculated because all samples but one stratified to the low risk group. The proneural G-CIMP negative group was not significant (HR = 1.15, 95% CI = 0.70–1.86, $p = 0.059$). The survival groups also had significantly different PFS by log-rank ($p = 9.91e{-}08$) (Figure 2F). There were 26 samples in the cohort with IDH1 mutations, only one of which stratified to the high-risk group, which suggests the signature is selecting for a subtype with already known survival differences.

The risk score was evaluated by fitting a Cox model incorporating the risk group and other factors involved in glioblastoma prognosis (gender, MGMT methylation, IDH mutation, patient subtype, extent of resection and KPS score). In each case, the score was significant and was not related to these factors (Supplementary Table S2).

We then calculated the risk score solely in the group of patients treated with the most common chemotherapy agent, temozolomide ($n = 219$). This group showed a high association between risk score and survival using log-rank ($p = 8.6e{-}04$) (Figure 3A). The power of the signature was compared to that of *MGMT* status by the log-rank test. In the 304 patients for whom *MGMT* methylation status was available (Brennan et al., 2013), multivariable Cox regression indicated the microRNA signature (HR = 1.88, CI = 1.42–2.48, $p = 9.4e{-}06$), which showed a 1.88-fold increased risk when stratified to the high-risk group, was more predictive than the *MGMT* methylation signature (HR = 1.47, CI = 1.12–1.93, $p = 0.006$), which showed a 1.47-fold increase in risk when *MGMT* is unmethylated. In the group treated with temozolomide only ($n = 219$) there was a 1.76-fold increase in risk by stratification to the low-risk group; this stratifies patients better than the *MGMT* signature, which shows a 1.65-fold increase in risk when stratified to the unmethylated group in the TCGA dataset.

3.4. Risk score validation in an independent dataset

Risk scores were calculated for an independent dataset of 20 glioblastoma samples (Supplemental Figure S3), with microRNA expression generated using qRT-PCR and was significantly associated with survival (HR = 10.7, $p = 0.036$). This patient group had an overall worse prognosis (80% died earlier than the expected median of 450 days) than those in the TCGA (70% died earlier than 450 days), and therefore, expecting more patients to fall into the high-risk group, the patients were dichotomized based on the 60th percentile ($0.76\Delta C_t$). This resulted in 12 patients in the high-risk group with a median survival of 6.27 months and 8 patients in the low-risk group with a median survival of 16 months. These groups predict survival using a one-sided log-rank test (HR = 3.01, $p = 0.045$) (Figure 3B).

3.5. Risk score assessment in lower grade glioma

Risk scores were also calculated for grade II and III gliomas ($n = 178$), using TCGA sequencing data. This was done using the 9 microRNAs and weighting derived in glioblastoma. The cohort was dichotomized into high- and low-risk groups using the median (−19541.96 reads per million) as a cut-off. As observed in the glioblastoma dataset, the score proved to be a significant predictor of survival using log-rank (Figure 3C, $p = 5.2e{-}03$) and in a Cox model with age (Group HR = 0.62, CI = 1.05–3.31, $p = 3.5e{-}02$; Age HR = 1.06, CI = 1.04–1.10, $p = 2.2e{-}07$). The low-risk group comprised of 44 grade II and 45 grade III samples; 22 were astrocytomas, 22 oligoastrogliomas and 45 oligodendrogliomas. The high-risk group comprised of 37 grade II samples and 51 grade III samples (1 not stated); 33 were astrocytomas, 25 oligoastrocytomas and 30 oligodendrogliomas.

3.6. Predicted targets of these microRNAs

Bioinformatic analysis was used to investigate targets of signature microRNAs to identify the associated pathways involved. Firstly, genes associated with long and short survival groups in glioblastoma were identified in TCGA. A total of 1154 genes were associated with short and 400 genes with long survival (Supplementary Figure S4).

Predicted interactions of the 9 microRNAs with the survival-associated genes were assessed in the Miranda (Miranda et al., 2006), Pictar (Krek et al., 2005) and Targetscan (Lewis et al., 2005) databases. This led to the identification of 10 significant microRNA/mRNA interactions with an inverse correlation of at least 0.25 across all glioblastoma samples (Table 3). Using DIANA miRPath (Vlachos et al., 2012) we identified the top pathways that the signature microRNAs are predicted to target. The most significant pathways identified included adherens junction, MAPK signaling, focal adhesion, axon guidance and WNT signaling (Supplementary Figure S5).

Targets implicated most strongly in patient survival were identified for miR-9, which showed a significant correlation with eight mRNAs. Correlation with FBN1 is shown in Figure 3D. In order to assess whether these may be functional targets, a glioblastoma cell line was transfected with a miR-9 mimic and the expression levels of the predicted targets were assessed using qPCR. *LMNA*, *WNT4*, *FBN1*, *P4HA2* and *SLC25A24* had significantly lower levels of expression when transfected with the mimic in comparison to a scrambled control (Figure 3E) suggesting miR-9 may directly target these mRNAs in glioblastoma cells.

Thus, bioinformatic analysis of signature microRNAs has identified potential targets and biological processes known

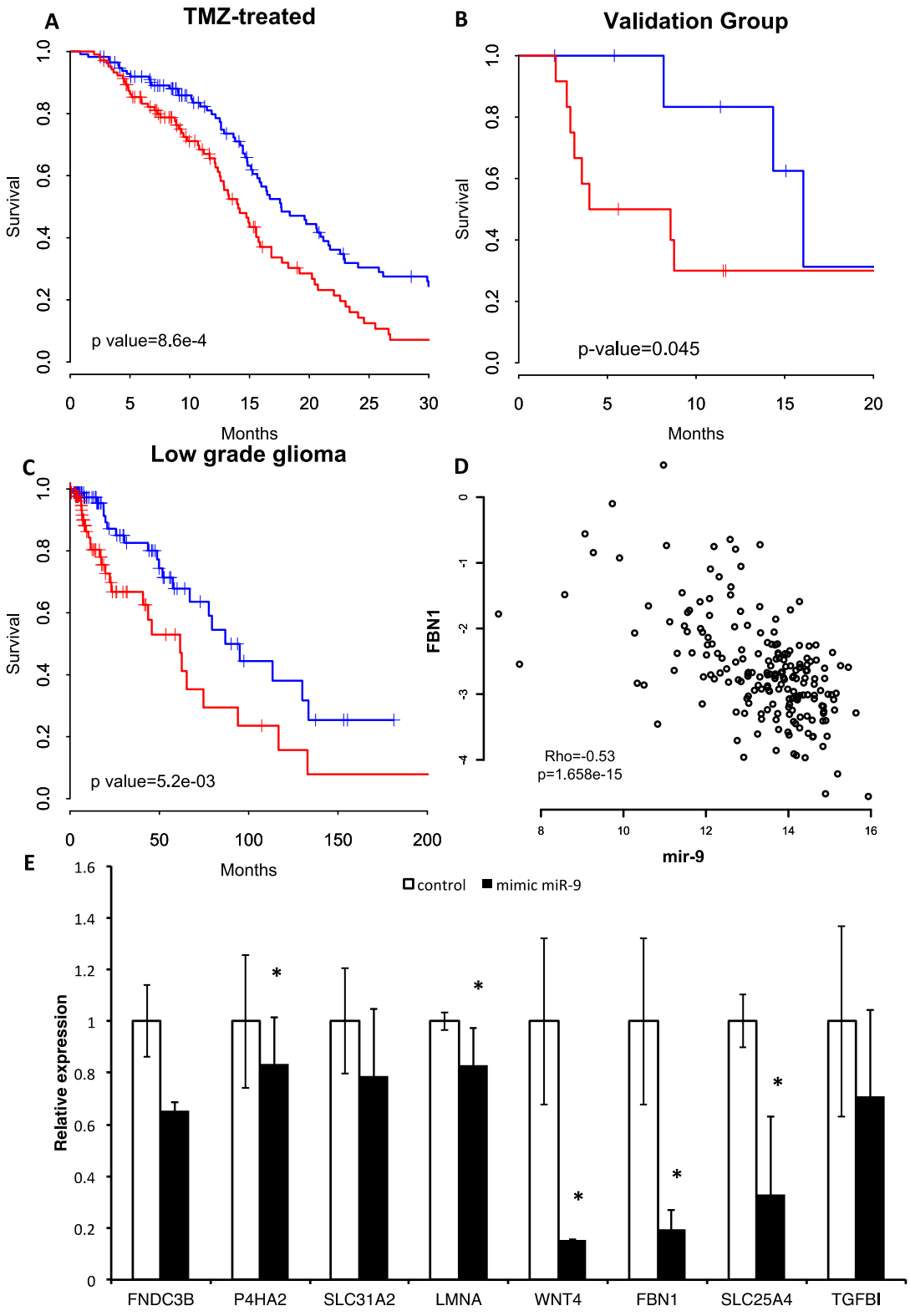

Figure 3 – Assessment of risk groups in TMZ treated patients, the validation cohort and lower grade glioma and correlation of *FBN1* with miR-9. A) The subgroup of patients treated with the chemotherapy agent temozolomide was significantly delineated using the signature. B) MicroRNA expression determined by qRT-PCR in an independent cohort of 20 glioblastomas stratified patients by survival based on the signature. C) MicroRNA sequencing data of 178 lower grade glioma samples (55 astrocytoma, 47 oligodendrocytoma, 75 oligodendroglioma, 1 not stated) significantly separated this cohort into high and low risk groups by log-rank. D) *FBN1* mRNA expression showed an inverse correlation of at least

Table 3 – Predicted target interactions of the signature microRNAs with significant correlation in expression. The ten interactions predicted between the 9-microRNA signature and the mRNAs identified to be involved in survival, which also showed a significant inverse correlation in expression of at least 0.25 across the patient set. Two of these mRNAs, *FBN1* and *TGFBI*, exhibited particularly high correlations in expression with miR-9 as well as significant ($p < 0.05$) differential expression between glioblastoma compared to non-tumor tissue.

MicroRNA	LASSO penalized coefficient (log 2)	Gene symbol	Gene change with increasing survival	Spearmans correlation	Fold difference in GBM to non-tumor tissue	*p*-Value of GBM/normal (FDR adjusted)
hsa-miR-9	**−0.032**	**TGFBI**	**4.499**	**−0.649**	**11.487**	**0.000**
hsa-miR-9	−0.032	P4HA2	2.527	−0.615	1.108	0.999
hsa-miR-9	**−0.032**	**FBN1**	**2.054**	**−0.53**	**1.808**	**0.001**
hsa-miR-222	0.162	KHDRBS2	**0.189**	−0.496	0.024	0.000
hsa-miR-9	−0.032	SLC25A24	3.574	−0.473	2.17	0.000
hsa-miR-9	−0.032	SLC31A2	2.384	−0.463	0.593	0.039
hsa-miR-9	−0.032	FNDC3B	2.171	−0.406	3.828	0.000
hsa-miR-182	−0.021	F13A1	10.982	−0.309	1.785	0.106
hsa-miR-9	−0.032	LMNA	2.034	−0.292	2.25	0.000
hsa-miR-9	−0.032	WNT4	2.038	−0.265	0.691	0.003

to be involved in glioblastoma biology, further supporting the relevance of the 9-microRNA signature.

4. Discussion

4.1. *The 9-microRNA signature is a molecular indicator of prognosis*

Using LASSO regression, this study has identified and independently validated a biologically relevant 9-microRNA signature that predicts survival in glioblastoma. The signature separates patients into high- and low-risk groups with respect to OS and PFS and may have clinical utility for decisions on patient management. The signature is valid in all glioblastoma subtypes except proneural G-CIMP negative tumors, and is linked to temozolomide response.

The independent dataset used here is relatively small and therefore confounding factors for patient age, treatment received and extent of resection could not be accounted for. Further validation, ideally prospective, and calculation of sensitivity and specificity, is required before this signature could be implemented clinically. The independent dataset results that were generated using qRT-PCR indicate that the signature can be implemented using techniques that would be more conducive to a clinical diagnostic laboratory and these are the methods that should be explored further. A limitation of this approach is that a different technique has been used for validation and therefore a single, defined cut-off could not be ascertained.

Prognostic signatures using microRNAs have been formulated previously in glioblastoma but these have not been validated or evaluated within different subgroups of the disease, or in relation to molecular characteristics of the disease (Kim et al., 2011; Niyazi et al., 2011; Srinivasan et al., 2011; Visani et al., 2013). A recent study identified prognostic microRNAs for each subtype of glioblastoma using TCGA data (Li et al., 2014) and five microRNAs in our signature overlap; miR-222, which they report predicts prognosis in classical and neural, miR-370 which predicts prognosis in neural and miR-34a, miR-145 and miR-182 which predict prognosis in the proneural non-G-CIMP group. Interestingly, 3/9 microRNAs in our signature are present in their model for proneural G-CIMP negative tumors yet our signature did not significantly stratify patients in this subtype.

The LASSO regression model was chosen to improve on other approaches by utilizing all 475 patients, and all microRNAs available to build the signature. This allows a small number of microRNAs for use in a diagnostic signature with maximal information but does not identify all predictors in the dataset involved in survival. This provides a signature with the prediction power similar, or better than, that of *MGMT* methylation. It must be noted however that MGMT methylation was assessed in an unselected population, with the Infinium methylation beadchip {Bady:2012jb}, which is not the gold standard employed in a diagnostic laboratory and therefore may lack sensitivity compared to clinical results. MGMT was also not assessed in the validation dataset due to lack of methylation data so this finding requires further confirmation. This signature has a manageable number of microRNAs for a prognostic indicator, and is well below the number of predictors employed in commercialized kits for other cancer signatures such as Mammaprint (Sorlie et al., 2001) and ms-14 (Cheang et al., 2009) in breast cancer.

4.2. *Roles of the microRNAs in the signature in glioma biology*

All microRNAs in this signature, with the exception of miR-370, have been previously associated with glioma biology

0.5 with miR-9 expression. E) Expression of the predicted targets following transfection of a miR-9 mimic into LN229 cells relative to a scrambled control. Significant decrease in expression (*t*-test, $p < 0.05$) was observed for *P4HA2*, *LMNA*, *WNT4*, *FBN1* and *SLC25A24* 48 h after transfection of the mimic. Results are representative of duplicate experiments.

(Fowler et al., 2011; Gabriely et al., 2011; Genovese et al., 2012; Kim et al., 2014; Mucaj et al., 2014; Rani et al., 2013; Song et al., 2012; Tan et al., 2012; Zhang et al., 2010) which has not been shown for previous glioblastoma microRNA signatures {Lakomy:2011ju}{Srinivasan:2011fh}{Zhang:2012iq}. Although miR-370 has not been reported to have a role in glioblastoma, it targets TGFB-RII (Lo et al., 2012), which has a role in glioblastoma cell growth and invasion (Kaminska et al., 2013). These studies suggest a potential role for miR-370 in glioma biology. Establishing a defined role for these microRNAs in glioma biology requires further work to determine the direct roles of these microRNAs in predicting prognosis of glioblastoma.

In addition to their established roles in glioma biology, 5 of the 9 signature microRNAs have been associated with sensitivity to temozolomide; miR-9 (Munoz et al., 2013), miR-145 (Yang et al., 2012), miR-148a (Hummel et al., 2011), miR-182 (Tang et al., 2013) and miR-222 (Chen et al., 2012). These observations suggest that the microRNA signature reflects roles in both tumor biology and treatment resistance, which combined lead to significant effects on patient survival.

4.3. *Translational relevance of the signature*

This prognostic signature has potential applicability to the clinic by stratifying patients, and identifying those less likely to respond to current treatments. The signature ultimately may facilitate confidence in treatment decisions and recognizing candidates for new therapies. It may be that the most powerful use of the signature is in combination with *MGMT* methylation status. Technologies such as the nanostring nCounter platform may provide highly accurate quantitative measurements of transcripts for tumor diagnosis as has been shown for medulloblastoma (Northcott et al., 2012), and is readily applicable to microRNA studies.

In conclusion, we have identified and validated a 9-microRNA-expression signature using biologically relevant markers of use in prediction of prognosis in glioblastoma. Analysis of targets of these microRNAs has identified potential key players in glioblastoma networks that could be targeted to combat the aggressive disease. The LASSO approach may be more broadly applicable in the identification of relevant microRNA and gene expression signatures in large datasets.

Funding

Josie Hayes is a Yorkshire Cancer Research (UK Registered Charity #516898) funded PhD student and consumables were funded by Cancer Research UK (UK Registered Charity #1089464). Dr Lawler was funded by a grant from Brain Tumour Research and Support across Yorkshire (UK Registered Charity #1095931)/Candlelighters (UK Registered Charity #1045077).

Conflict of interest

The authors have no conflicts of interest to declare.

Appendix A. Supplementary material

Supplementary data related to this article can be found online at http://dx.doi.org/10.1016/j.molonc.2014.11.004.

REFERENCES

Alencar, A.J., Malumbres, R., Kozloski, G.A., Advani, R., Talreja, N., Chinichian, S., Briones, J., Natkunam, Y., Sehn, L.H., Gascoyne, R.D., Tibshirani, R., Lossos, I.S., 2011. MicroRNAs are independent predictors of outcome in diffuse large B-cell lymphoma patients treated with R-CHOP. Clin. Cancer Res. 17, 4125–4135. http://dx.doi.org/10.1158/1078-0432.CCR-11-0224.

Brennan, C.W., Verhaak, R.G.W., McKenna, A., Campos, B., Noushmehr, H., Salama, S.R., et al., 2013. The somatic genomic landscape of glioblastoma. Cell 155, 462–477. http://dx.doi.org/10.1016/j.cell.2013.09.034.

Calin, G.A., Croce, C.M., 2006. MicroRNA signatures in human cancers. Nat. Rev. Cancer 6, 857–866. http://dx.doi.org/10.1038/nrc1997.

Chaichana, K.L., Pendleton, C., Chambless, L., Camara-Quintana, J., Nathan, J.K., Hassam-Malani, L., Li, G., Harsh 4th, G.R., Thompson, R.C., Lim, M., Quinones-Hinojosa, A., 2013. Multi-institutional validation of a preoperative scoring system which predicts survival for patients with glioblastoma. J. Clin. Neurosci. 20, 1422–1426. http://dx.doi.org/10.1016/j.jocn.2013.02.007.

Cheang, M.C.U., Chia, S.K., Voduc, D., Gao, D., Leung, S., Snider, J., Watson, M., Davies, S., Bernard, P.S., Parker, J.S., Perou, C.M., Ellis, M.J., Nielsen, T.O., 2009. Ki67 index, HER2 status, and prognosis of patients with luminal B breast cancer. J. Natl. Cancer Inst. 101, 736–750. http://dx.doi.org/10.1093/jnci/djp082.

Chen, L., Zhang, J., Han, L., Zhang, A., Zhang, C., Zheng, Y., Jiang, T., Pu, P., Jiang, C., Kang, C., 2012. Downregulation of miR-221/222 sensitizes glioma cells to temozolomide by regulating apoptosis independently of p53 status. Oncol. Rep. 27, 854–860. http://dx.doi.org/10.3892/or.2011.1535.

Favero, F., v2.14. RmiR: Package to Work with miRNAs and miRNA Targets with R.

Fowler, A., Thomson, D., Giles, K., Maleki, S., Mreich, E., Wheeler, H., Leedman, P., Biggs, M., Cook, R., Little, N., Robinson, B., McDonald, K., 2011. miR-124a is frequently down-regulated in glioblastoma and is involved in migration and invasion. Eur. J. Cancer 47, 953–963. http://dx.doi.org/10.1016/j.ejca.2010.11.026.

Gabriely, G., Yi, M., Narayan, R.S., Niers, J.M., Wurdinger, T., Imitola, J., Ligon, K.L., Kesari, S., Esau, C., Stephens, R.M., Tannous, B.A., Krichevsky, A.M., 2011. Human glioma growth is controlled by microRNA-10b. Cancer Res. 71, 3563–3572. http://dx.doi.org/10.1158/0008-5472.CAN-10-3568.

Genovese, G., Ergun, A., Shukla, S.A., Campos, B., Hanna, J., Ghosh, P., Quayle, S.N., Rai, K., Colla, S., Ying, H., Wu, C.J., Sarkar, S., Xiao, Y., Zhang, J., Zhang, H., Kwong, L., Dunn, K., Wiedemeyer, W.R., Brennan, C., Zheng, H., Rimm, D.L., Collins, J.J., Chin, L., 2012. microRNA regulatory network inference identifies miR-34a as a novel regulator of TGF-signaling in glioblastoma. Cancer Discov. 2, 736–749. http://dx.doi.org/10.1158/2159-8290.CD-12-0111.

Goeman, J.J., 2012. Penalized R Package, Version 0.9-42.

Griffiths-Jones, S., Saini, H.K., Van Dongen, S., Enright, A.J., 2008. miRBase: tools for microRNA genomics. Nucleic Acids Res. 36, D154–D158.

Hayes, J., Peruzzi, P.P., Lawler, S., 2014. MicroRNAs in cancer: biomarkers, functions and therapy. Trends Mol. Med. 20, 460–469. http://dx.doi.org/10.1016/j.molmed.2014.06.005.

Hummel, R., Watson, D.I., Smith, C., Kist, J., Michael, M.Z., Haier, J., Hussey, D.J., 2011. Mir-148a improves response to chemotherapy in sensitive and resistant oesophageal adenocarcinoma and squamous cell carcinoma cells. J. Gastrointest. Surg. 15, 429–438. http://dx.doi.org/10.1007/s11605-011-1418-9.

Kaminska, B., Kocyk, M., Kijewska, M., 2013. TGF beta signaling and its role in glioma pathogenesis. Adv. Exp. Med. Biol. 986, 171–187. http://dx.doi.org/10.1007/978-94-007-4719-7_9.

Kim, J., Zhang, Y., Skalski, M., Hayes, J., Kefas, B., Schiff, D., Purow, B., Parsons, S., Lawler, S., Abounader, R., 2014. microRNA-148a is a prognostic oncomiR that targets MIG6 and BIM to regulate EGFR and apoptosis in glioblastoma. Cancer Res. 74, 1541–1553.

Kim, T.-M., Huang, W., Park, R., Park, P.J., Johnson, M.D., 2011. A developmental taxonomy of glioblastoma defined and maintained by MicroRNAs. Cancer Res. 71, 3387–3399. http://dx.doi.org/10.1158/0008-5472.CAN-10-4117.

Krek, A., Grun, D., Poy, M.N., Wolf, R., Rosenberg, L., Epstein, E.J., MacMenamin, P., da Piedade, I., Gunsalus, K.C., Stoffel, M., Rajewsky, N., 2005. Combinatorial microRNA target predictions. Nat. Genet. 37, 495–500. http://dx.doi.org/10.1038/ng1536.

Krex, D., Klink, B., Hartmann, C., Deimling, von A., Pietsch, T., Simon, M., Sabel, M., Steinbach, J.P., Heese, O., Reifenberger, G., Weller, M., Schackert, G., Network, F.T.G.G., 2007. Long-term survival with glioblastoma multiforme. Brain 130, 2596–2606.

Krol, J., Loedige, I., Filipowicz, W., 2010. The widespread regulation of microRNA biogenesis, function and decay. Nat. Rev. Genet. 11, 597–610. http://dx.doi.org/10.1038/nrg2843.

Lang, M.-F., Yang, S., Zhao, C., Sun, G., Murai, K., Wu, X., Wang, J., Gao, H., Brown, C.E., Liu, X., Zhou, J., Peng, L., Rossi, J.J., Shi, Y., 2012. Genome-wide profiling identified a set of miRNAs that are differentially expressed in glioblastoma stem cells and normal neural stem cells. PLoS ONE 7, e36248. http://dx.doi.org/10.1371/journal.pone.0036248.t002.

Lewis, B.P., Burge, C.B., Bartel, D.P., 2005. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. Cell 120, 15–20. http://dx.doi.org/10.1016/j.cell.2004.12.035.

Li, R., Gao, K., Luo, H., Wang, X., Shi, Y., Dong, Q., Luan, W., You, Y., 2014. Identification of intrinsic subtype-specific prognostic microRNAs in primary glioblastoma. J. Exp. Clin. Cancer Res. 33, 1–6. http://dx.doi.org/10.1186/1756-9966-33-9.

Lo, S.-S., Hung, P.-S., Chen, J.-H., Tu, H.-F., Fang, W.-L., Chen, C.Y., Chen, W.-T., Gong, N.-R., Wu, C.-W., 2012. Overexpression of miR-370 and downregulation of its novel target TGFbeta-RII contribute to the progression of gastric carcinoma. Oncogene 31, 226–237. http://dx.doi.org/10.1038/onc.2011.226.

Louis, D.N., Ohgaki, H., Wiestler, O.D., Cavenee, W.K., Burger, P.C., Jouvet, A., Scheithauer, B.W., Kleihues, P., 2007. The 2007 WHO classification of tumours of the central nervous system. Acta Neuropathol. 114, 97–109. http://dx.doi.org/10.1007/s00401-007-0243-4.

Miranda, K.C., Huynh, T., Tay, Y., Ang, Y.-S., Tam, W.-L., Thomson, A.M., Lim, B., Rigoutsos, I., 2006. A pattern-based method for the identification of MicroRNA binding sites and their corresponding heteroduplexes. Cell 126, 1203–1217. http://dx.doi.org/10.1016/j.cell.2006.07.031.

Mizoguchi, M., Guan, Y., Yoshimoto, K., Hata, N., Amano, T., Nakamizo, A., Sasaki, T., 2012. MicroRNAs in human malignant gliomas. J. Oncol. 2012, 1–7. http://dx.doi.org/10.1523/JNEUROSCI.4966-09.2009.

Mucaj, V., Lee, S.S., Skuli, N., Giannoukos, D.N., Qiu, B., Eisinger-Mathason, T.S.K., Nakazawa, M.S., Shay, J.E.S., Gopal, P.P., Venneti, S., Lal, P., Minn, A.J., Simon, M.C., Mathew, L.K., 2014. MicroRNA-124 expression counteracts pro-survival stress responses in glioblastoma. Oncogene. http://dx.doi.org/10.1038/onc.2014.168.

Munoz, J.L., Bliss, S.A., Greco, S.J., Ramkissoon, S.H., Ligon, K.L., Rameshwar, P., 2013. Delivery of functional anti-miR-9 by mesenchymal stem cell-derived exosomes to glioblastoma multiforme cells conferred chemosensitivity. Mol. Ther. Nucl. Acids. http://dx.doi.org/10.1038/mtna.2013.60.

Niyazi, M., Zehentmayr, F., Niemöller, O.M., Eigenbrod, S., Kretzschmar, H., Schulze-Osthoff, K., Tonn, J.-C., Atkinson, M., Mörtl, S., Belka, C., 2011. MiRNA expression patterns predict survival in glioblastoma. Radiat. Oncol. 6, 153. http://dx.doi.org/10.1186/1748-717X-6-153.

Northcott, P.A., Shih, D.J.H., Remke, M., Cho, Y.-J., Kool, M., Hawkins, C., Eberhart, C.G., Dubuc, A., Guettouche, T., Cardentey, Y., Bouffet, E., Pomeroy, S.L., Marra, M., Malkin, D., Rutka, J.T., Korshunov, A., Pfister, S., Taylor, M.D., 2012. Rapid, reliable, and reproducible molecular sub-grouping of clinical medulloblastoma samples. Acta Neuropathol. 123, 615–626. http://dx.doi.org/10.1007/s00401-011-0899-7.

Ohgaki, H., Kleihues, P., 2007. Genetic pathways to primary and secondary glioblastoma. Am. J. Pathol. 170, 1445–1453. http://dx.doi.org/10.2353/ajpath.2007.070011.

Parsons, D.W., Jones, S., Zhang, X., Lin, J.C.H., Leary, R.J., Angenendt, P., Mankoo, P., Carter, H., Siu, I.-M., Gallia, G.L., Olivi, A., McLendon, R., Rasheed, B.A., Keir, S., Nikolskaya, T., Nikolsky, Y., Busam, D.A., Tekleab, H., Diaz, L.A., Hartigan, J., Smith, D.R., Strausberg, R.L., Marie, S.K.N., Shinjo, S.M.O., Yan, H., Riggins, G.J., Bigner, D.D., Karchin, R., Papadopoulos, N., Parmigiani, G., Vogelstein, B., Velculescu, V.E., Kinzler, K.W., 2008. An integrated genomic analysis of human glioblastoma multiforme. Science 321, 1807–1812. http://dx.doi.org/10.1126/science.1164382.

Rani, S.B., Rathod, S.S., Karthik, S., Kaur, N., Muzumdar, D., Shiras, A.S., 2013. MiR-145 functions as a tumor-suppressive RNA by targeting Sox9 and adducin 3 in human glioma cells. Neuro-oncology 15, 1302–1316. http://dx.doi.org/10.1093/neuonc/not090.

Riemenschneider, M.J., Jeuken, J.W.M., Wesseling, P., Reifenberger, G., 2010. Molecular diagnostics of gliomas: state of the art. Acta Neuropathol. 120, 567–584. http://dx.doi.org/10.1007/s00401-010-0736-4.

Song, L., Liu, L., Wu, Z., Li, Y., Ying, Z., Lin, C., Wu, J., Hu, B., Cheng, S.-Y., Li, M., Li, J., 2012. TGF-beta induces miR-182 to sustain NF-kappaB activation in glioma subsets. J. Clin. Invest. 122, 3563–3578. http://dx.doi.org/10.1172/JCI62339.

Sorlie, T., Perou, C.M., Tibshirani, R., Aas, T., Geisler, S., Johnsen, H., Hastie, T., Eisen, M.B., van de Rijn, M., Jeffrey, S.S., Thorsen, T., Quist, H., Matese, J.C., Brown, P.O., Botstein, D., Lonning, P.E., Borresen-Dale, A.L., 2001. Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. Proc. Natl. Acad. Sci. U. S. A. 98, 10869–10874.

Srinivasan, S., Patric, I.R.P., Somasundaram, K., 2011. A ten-microRNA expression signature predicts survival in glioblastoma. PLoS ONE 6, e17438. http://dx.doi.org/10.1371/journal.pone.0017438.

Stupp, R., Roila, F., 2009. Malignant glioma: ESMO clinical recommendations for diagnosis, treatment and follow-up. Ann. Oncol. 20, 126–128. http://dx.doi.org/10.1093/annonc/mdp151.

Tan, X., Wang, S., Yang, B., Zhu, L., Yin, B., Chao, T., Zhao, J., Yuan, J., Qiang, B., Peng, X., 2012. The CREB-miR-9 negative feedback minicircuitry coordinates the migration and

proliferation of glioma cells. PLoS ONE 7, e49570. http://dx.doi.org/10.1371/journal.pone.0049570.s006.

Tang, H., Bian, Y., Tu, C., Wang, Z., Yu, Z., Liu, Q., Xu, G., Wu, M., Li, G., 2013. The miR-183/96/182 cluster regulates oxidative apoptosis and sensitizes cells to chemotherapy in gliomas. Curr. Cancer Drug Targets 13, 221–231.

The Cancer Genome Atlas – Data Portal [WWW Document]. URL: http://tcga-data.nci.nih.gov/ (accessed 24.04.13).

Tibshirani, R., 1996. Regression shrinkage and selection via the Lasso. J. R. Stat. Soc. Ser. B-Methodol. 58, 267–288. http://dx.doi.org/10.1021/np300902g.

Visani, M., de Biase, D., Marucci, G., Taccioli, C., Baruzzi, A., Pession, A.the PERNO Study group, 2013. Definition of miRNAs expression profile in glioblastoma samples: the relevance of non-neoplastic brain reference. PLoS ONE 8, e55314. http://dx.doi.org/10.1371/journal.pone.0055314.s002.

Vlachos, I.S., Kostoulas, N., Vergoulis, T., Georgakilas, G., Reczko, M., Maragkakis, M., Paraskevopoulou, M.D., Prionidis, K., Dalamagas, T., Hatzigeorgiou, A.G., 2012. DIANA miRPath v.2.0: investigating the combinatorial effect of microRNAs in pathways. Nucleic Acids Res. 40, W498–W504. http://dx.doi.org/10.1093/nar/gks494.

Volinia, S., Calin, G.A., Liu, C.-G., Ambs, S., Cimmino, A., Petrocca, F., Visone, R., Iorio, M., Roldo, C., Ferracin, M., Prueitt, R.L., Yanaihara, N., Lanza, G., Scarpa, A., Vecchione, A., Negrini, M., Harris, C.C., Croce, C.M., 2006. A microRNA expression signature of human solid tumors defines cancer gene targets. Proc. Natl. Acad. Sci. U. S. A. 103, 2257–2261. http://dx.doi.org/10.1073/pnas.0510565103.

Yang, Y.-P., Chien, Y., Chiou, G.-Y., Cherng, J.-Y., Wang, M.-L., Lo, W.-L., Chang, Y.-L., Huang, P.-I., Chen, Y.-W., Shih, Y.-H., Chen, M.-T., Chiou, S.-H., 2012. Inhibition of cancer stem cell-like properties and reduced chemoradioresistance of glioblastoma using microRNA145 with cationic polyurethane-short branch PEI. Biomaterials 33, 1462–1476. http://dx.doi.org/10.1016/j.biomaterials.2011.10.071.

Zhang, C.-Z., Zhang, J.-X., Zhang, A.-L., Shi, Z.-D., Han, L., Jia, Z.-F., Yang, W.-D., Wang, G.-X., Jiang, T., You, Y.-P., Pu, P.-Y., Cheng, J.-Q., Kang, C.-S., 2010. MiR-221 and miR-222 target PUMA to induce cell survival in glioblastoma. Mol. Cancer 9, 229. http://dx.doi.org/10.1186/1476-4598-9-229.